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THE BOTANICAL REVIEW

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THE INTERNAL MECHANISM OF SPECIATION IN *GOSSYPIUM*

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INTRODUCTION

The last twenty years have seen great advances in the cytogenetics and taxonomy of the genus *Gossypium*. From the taxonomic point of view, controversial questions are now restricted to minor differences of opinion on relative taxonomic rank, while the major evolutionary pattern seems to be generally agreed upon. One outstanding major problem still remains, *viz.*, the origin of the amphidiploid species. Although there is general agreement on the parentage of the amphidiploids, there is a sharp cleavage of opinion as to the time when amphidiploidy occurred. Whether a satisfactory solution of this problem will be reached is to a large extent in the hands of the archaeologist and palaeontologist, though the cytologist, too, may be able to make pertinent contributions.

Cytogenetic research in cotton is now entering a new phase. In the absence of new and revolutionary techniques it seems likely that the immediate future will be concerned with the consolidation of knowledge already obtained and with attempts to apply that knowledge to practical ends—improvement of the cotton crop. One may expect a shift in the direction of research, particular emphasis being placed on the transference of potentially valuable economic characters from wild to cultivated species. It is therefore particularly important at this point to be sure that the theory of interspecific differentiation, which has been developed during the past twenty years, rests on a firm basis, since the success or failure of future cotton breeding programs may depend almost entirely on it.

The fact that a theory is generally accepted and has formed a keystone in the building of knowledge does not guarantee that the theory is correct. Nor, on the other hand, does it necessarily follow that all work based on the assumption of its being correct

is invalidated if the theory eventually proves to be wrong. A theory may be right for one set of data and wrong for another—the atomic theory is a striking example. It is important, however, to avoid converting a theory into a doctrine after it has ceased to offer a convincing explanation of more recent discoveries. If a theory is not elastic enough to embrace new data, it must be modified or discarded. It is the purpose of this paper to re-examine the commonly accepted theory of speciation in *Gossypium* and to point out certain genetic, cytological and breeding phenomena which it does not explain satisfactorily. In the writer's opinion these phenomena, while they do not necessarily invalidate the broader aspects of the theory, do show that it is incomplete and suggest a possible form of modification.

THE THEORY OF MULTIPLE GENE SUBSTITUTION AS A BASIS FOR SPECIATION

When the cultivated amphidiploid species, *G. barbadense* L. and *G. hirsutum* L., are crossed together, the hybrid exhibits heterosis to a marked degree and is apparently fully fertile. Meiosis in the F_1 pollen mother cell is normal insofar as regular bivalent pairing is acceptable as a criterion of normality. The F_2 generation, however, always shows a considerable depression in vigor; weak seedlings, sterile, and physiologically unbalanced types being common. The types which most nearly approach the F_1 in appearance again produce a medley of unthrifty types in the following (F_3) generation. Only those types which resemble most closely one or other of the parental species become stabilized under human or natural selection. A similar situation is found in hybrid progenies of the diploid cultivated species, *G. arboreum* L. and *G. herbaceum* L.

On the basis of his genetic studies of *barbadense* × *hirsutum* hybrids, Harland (15) first suggested an explanation of this "interspecific breakdown": "... The first step in the evolution of a species is the production of new multiple allelomorphs which differ from the old in their capability of being influenced by new modifier complexes, thus rendering possible the building up of new characters through natural selection of plus modifiers operating in the given direction". "... the modifiers really constitute the species and ... as a consequence of geographical isolation and nat-

ural selection evolution takes place by the evolution of new genes, by the production of new co-ordinated modifier systems and by the elaboration of new dominance mechanisms".

That each species has its characteristic "architecture" and behaves as a genetically co-ordinated system has been amply verified in *Gossypium* by Harland's own work and that of other investigators. His theory of speciation has proved to be a most valuable tool in the analysis of species relationships, without which a satisfactory classification would have been impossible. But in the writer's opinion it is questionable that the differentiation of species can be attributed primarily to the substitution of one "modifying complex" by another, unless the term "modifier" is used in a very specialized sense. Following the generally accepted usage of the term, a modifier is a gene which modifies the expression of another, the effect of the modifier being small, in comparison with the effect of the gene which it modifies, on the character under study. In that case the substitution of one modifier by another could involve only the substitution of each modifier by an allele at each corresponding locus, and if speciation depends primarily on this process, there is no valid reason why "good" daughter species should not remain cytologically homologous, locus by locus, throughout their respective chromosome sets. Any further differentiation would involve some degree of structural change. Harland apparently accepted this restriction, though, as will be shown later, evidence obtained in recent years is not compatible with it.

From his studies of interspecific hybrids involving the cultivated Asiatic species, *G. arboreum* L. and *G. herbaceum* L., and the wild African species, *G. anomalum* Wawra et Peyr., Silow (42, 43) considered that these species had evolved in a manner almost identical with that suggested by Harland to account for differences between *hirsutum* and *barbadense*. The F_1 hybrid, *arboreum* × *herbaceum*, shows normal bivalent pairing and is apparently fully fertile, but, as in the *hirstum* × *barbadense* cross, the F_2 consists of a range of types, most of which are inferior to each parent. The F_1 hybrids, *arboreum* × *anomalum* and *herbaceum* × *anomalum*, are only weakly fertile, and meiotic figures in their pollen mother cells show clear evidence of structural differentiation (5, 48). Nevertheless, Silow found that these hybrids

were sufficiently fertile to enable him to undertake a very comprehensive study of the comparative genetics of the parent species. His conclusions agreed with Harland's: "... there is not any striking difference between the two cultivated Asiatic species as far as their main gene constitution is concerned. Practically all the difference lies in the modifier systems ...".

Anomalum was found to differ more widely from *arboreum* and *herbaceum* in respect to "main gene" constitution than these species differ from each other, and in modifier constitution it was "strikingly distinct from the two cultivated species, the hybrid progenies showing very much wider segregation than do crosses between the latter species". Silow's studies establish very definitely that the same basic mechanism is responsible for the differentiation of all species of *Gossypium* which are at least partially interfertile, and provide strong presumptive evidence that the same process has continued through the genus as a whole. It may be seriously questioned, however, that multiple gene substitution is as adequate a basic mechanism as he and many others, including the present writer, have supposed. In this paper an attempt will be made to show how the data accumulated in support of the hypothesis are capable of a different interpretation.

CRYPTIC STRUCTURAL DIFFERENTIATION AS A BASIS FOR SPECIATION

According to the theory of speciation by multiple gene substitution, the gross structural differentiation apparent in sterile or partially sterile hybrids must represent a secondary superimposed mechanism which may play a significant part in the isolation of species, but which cannot be directly concerned with the initiation of species differences. An exactly opposite point of view is held by Goldschmidt (10) who has suggested that species differences in *Gossypium*, among other genera, are attributable to different reaction systems determined by the structural patterns of whole chromosomes. *Is it necessary to accept either of these extreme hypotheses?* The writer first suggested (54) that genic and structural differentiation in *Gossypium* need not be considered as independent processes, and that a level of small scale structural differentiation could be envisaged which would be practically indistinguishable from purely genetic differentiation: "The frequency of *gross* structural changes (*i.e.*, inversions or changes in

homology of sufficient magnitude to produce bridges) in interspecific hybrids . . . suggests the likelihood that *small* structural changes also occur. These cannot be detected cytologically though their presence may be inferred from the restricted chromosome pairing in interspecific hybrids. In crosses between interfertile species they will behave as Mendelian units and hence will be indistinguishable from true gene mutations. Small structural changes would account for the continuity of the process of differentiation which is seen at all levels of speciation".

That sterility in intraspecific hybrids, or in hybrids between closely related species, is probably attributable to small scale structural differentiation which would not necessarily be detectable cytologically was concluded earlier by Sax (40) and Muntzing (36) as a result of their studies in *Campsis* and *Galeopsis*, respectively. There is therefore a reasonable precedent for considering the possibility of a similar situation in *Gossypium* where, as in the above named genera, the chromosomes are small and few chiasmata per bivalent are produced at meiosis (46). Hutchinson and Stephens (27) recognized that there was no good reason to expect any sharp distinction between so-called "point mutation" and gross structural differentiation—the most likely situation being an intergrading series linking these two extreme cases. In the middle of the range it is likely that many small structural changes would be mistaken for simple allelic differences on the basis of breeding behavior. The difficulty of critical discrimination, even in the most favorable material (*Drosophila* salivary glands), has been emphasized by Raffel and Muller (38).

Stebbins (50, 51) has suggested the convenient term "cryptic structural differentiation" to cover the situation outlined above. From a general review of similar cases he has inferred that cryptic structural differentiation is commonly associated with speciation: "There is . . . a good deal of evidence now available that a large proportion, if not a majority of the structural differences between genomes, involve segments so small that they do not give the typical meiotic figurations such as multivalents and bridge fragments in structural hybrids. . . . Many of the numerous examples now known of diploid hybrids that are partly or wholly sterile in spite of nearly regular meiosis are best explained on the basis of the phenomenon of cryptic structural hybridity".

The present writer would go even further than this and suggest

that in small chromosome types the production of markedly inferior F_2 progenies from fully fertile interspecific hybrids may also be attributable to cryptic structural hybridity, *i.e.*, to crossing-over and segregation of only partially homologous chromatids, leading to the production of deficient or otherwise unbalanced zygotes which would not necessarily be lethal nor completely sterile. In order for such an interpretation to hold it would of course be necessary to assume (*a*) that small deficiencies would not always behave as pollen lethals and (*b*) that crossing-over would occur fairly frequently between only partially homologous chromatids. With regard to (*a*), McClintock's evidence in corn (34) that a terminal deficiency so extensive as to include a knob, heterochromatic segment and half a chromomere in the ninth chromosome can be transmitted through the pollen removes any reasonable doubt that small deficiences can be carried in viable gametes. Furthermore, her work suggests that many so-called recessive mutants which are non-lethal may in fact be small deficiences which would be detectable only in very favorable material, *e.g.*, terminal deficiences in large chromosome types. With regard to (*b*) above, important information has been obtained at this station by Dr. M. S. Brown, which shows fairly conclusively that pairing between demonstrably non-homologous chromosomes can produce fertile gametes. Her evidence (8) in relation to the data of other investigators is summarized in Tables I and II. Referring to the tables, it can be seen that when the diploid *anomalum* (B genome) is crossed with the diploid *G. thurberi* Tod. (D_1 genome) the F_1 hybrid is completely sterile, and its meiotic figures show clear evidence of gross structural differentiation. The diploid *arboreum* (A_2 genome) gives a completely sterile hybrid on crossing with *thurberi*, and a partly sterile hybrid on crossing with *anomalum*. Colchicine synthesized amphidiploids, *arboreum* × *thurberi* ($2(A_2D_1)$) and *arboreum* × *anomalum* ($2(A_2B)$), are partly fertile and give preponderately bivalent pairing, indicating that pairing is chiefly between homologous chromosomes. But when the two amphidiploids are crossed together, their hybrid (A_2D_1/A_2B) is partly fertile, which is most readily explained as a result of regular bivalent pairing between the A_2 genomes and as a degree of regular pairing between the non-homologous B and D_1 genomes. An exactly similar situation was found in the cross (*arboreum* × *stocksii*) × (*arboreum* ×

anomalum), the multiple hybrid, A_2B/A_2E, being fertile, although the diploid hybrid, *anomalum* (B) × *stocksii* (E), was weak, unthrifty and sterile (47). The most likely explanation of the unexpected fertility obtained in these multiple hybrids is that the deficient or otherwise unbalanced chromatids resulting from crossing-over between B/D and B/E non-homologous "pairs", respectively, are covered by the presence of similar but not homol-

TABLE I

MEIOTIC BEHAVIOR AND FERTILITY OF F_1 HYBRIDS BETWEEN CERTAIN DIPLOID SPECIES OF *Gossypium*. GENOME CONSTITUTION INDICATED BY APPROPRIATE LETTERS ACCORDING TO BEASLEY'S SYSTEM

F_1 hybrid	Genome constitution	Metaphase I	Fertility	Authority
1. *arboreum* × *thurberi*	A_2D_1	Half of total chromosomes associated in pairs. Obvious structural differences.	Sterile	Skovsted (48) Webber (64) Beasley (5)
2. *arboreum* × *anomalum*	A_2B	Over three quarters of total chromosomes associated in pairs. A few structural differences.	10% fertile	Skovsted (48) Silow (42) Beasley (5) Afzal *et al.* (2)
3. *anomalum* × *thurberi*	BD	Less than half of total chromosomes associated in pairs.	Sterile	Skovsted (48)
4. *arboreum* × *stocksii*	A_2E	Less than half of total chromosomes associated in pairs.	Sterile	Skovsted (48) Webber (64) Abraham (1) Beasley (5)
5. *anomalum* × *stocksii*	BE	1 nucleus analyzed by Beasley gave 5II 16I.	Sterile	Hutchinson cited Skovsted (47) Beasley (unpublished)

ogous chromatid segments in the normally pairing A_2 genomes. Deficiencies would therefore be masked in the resulting gametes by the normally balanced A_2 chromosome set. In the direct diploid × diploid crosses the deficiencies would be exposed and, presumably, since the resulting hybrids are sterile or partly sterile, would act as gametic lethals. Whether this is the correct interpretation or not, the foregoing data have an extremely

important bearing on the reliance one can place on "normal" bivalent pairing as a measure of cytological homology. Let us consider the situation which would exist if the amphidiploids cited had occurred in nature and if the original diploids had been lost. Since the amphidiploids give a partially fertile hybrid, we should almost certainly assume a reasonably close homology between their respective genomes—perhaps even suppose that their speciation was in the main associated with multiple gene substitution. In

TABLE II

MEIOTIC BEHAVIOR AND FERTILITY OF SYNTHETIC TETRAPLOIDS OBTAINED BY COLCHICINE TREATMENT OF CERTAIN OF THE DIPLOID HYBRIDS LISTED IN TABLE I

	Synthetic tetraploid	Genome constitution	Metaphase I	Fertility	Authority
1.	*arboreum* × *thurberi*	$2(A_2D_1)$	Approximates to "normal" bivalent pairing.	Female fertile, male sterile	Beasley (5) Harland (21)
2.	*arboreum* × *anomalum*	$2(A_2B)$	Over three quarters of total chromosomes associated as bivalents.	Partly fertile male and female	Iyengar (28) Stephens (54) Brown (8)
3.	*arboreum* × *stocksii*	$2(A_2E)$	Over three quarters of total chromosomes associated as bivalents. (Brown unpublished).	Partly fertile male and female	Stephens and Cassidy (60) Brown (8)
	F_1 (1) × (2)	A_2D_1/A_2B	.....	Partly fertile	Brown (8)
	F_1 (2) × (3)	A_2B/A_2E	.....	Partly fertile	Brown (8)

short we should apply the same reasoning as has already been applied to a similar situation, *viz.*, the differentiation of *hirsutum* and *barbadense*. But in the case of the synthetic amphidiploids we are fortunate in having the evidence of pairing behavior of the component subgenomes, *i.e.*, the original diploids, which indicates clearly that there is considerable structural differentiation. We can therefore no longer assume with any confidence that fertility of the *hirsutum* × *barbadense* hybrid is indicative of structural homology between the parent species.

It might be argued that the above considerations are applicable only to hybrids between polyploid species, where the possibility of one sub-genome masking deficiencies in the other sub-genome would be at a maximum. In *Gossypium*, however, this cannot be considered a valid objection. There is considerable evidence, both cytological (5, 29, 46, 48, 52, 54) and genetic (42), that the "diploid" species, *arboreum* and *herbaceum*, either are themselves secondary polyploids or at least contain several segmental duplications.

EVIDENCE OF CRYPTIC STRUCTURAL DIFFERENTIATION IN *Gossypium*

The very term "cryptic structural differentiation" implies a difficulty in discriminating between this mechanism of speciation and a mechanism based on purely genetic differentiation. Furthermore, the general acceptance of the multiple gene substitution theory has not stimulated research in the development of critical tests of its validity. Evidence which has a bearing on the problem has to a large extent been collected for other purposes, and its significance has therefore escaped notice or else been underestimated. All that can be attempted here is to present rather scattered evidence obtained from cytological, genetic and breeding data, which in the writer's opinion is more consistent with structural differentiation than purely genetic differentiation, and to suggest methods of obtaining more satisfactory data.

Cytological Evidence

Bivalent Pairing in Fertile Interspecific Hybrids. Skovsted (46) noted that in pure species of Asiatic cottons, the maximum number of chiasmata formed from diplotene to first metaphase was three per bivalent, two being the most common number. In the interspecific hybrid, *arboreum* × *herbaceum*, the chiasmata frequency was less than in either parent species. Thus, with regard to the pachytene chromosomes, he states: "... it is the writer's impression that the doubleness of the pachytene strands was much more easily observed in the hybrid than in the pure species due to the fact that the threads were separated over larger distances".

Of the later stages (diakinesis and first metaphase) he says: "... all the partners must have formed at least two chiasmas,

which seems to be a common phenomenon in a normal diploid Asiatic cotton. In the interspecific hybrid the number of chiasmas is less, so that both rods and rings are common. A single pair is characterized by a conjugation so loose that most likely the chiasma is occasionally not formed which will result in univalent chromosomes as the second division indicates".

In F_1 hybrids from similar material, Webber (63) found occasional lagging bivalents and numerically unbalanced second metaphase plates. Beasley (5) frequently found a bridge and fragment at first and second anaphases in the interspecific hybrid, and also noted figures with four chromosomes associated.

In the interspecific hybrid, ***hirsutum*** × ***barbadense***, both Webber and Beasley (*loc. cit.*) were of the opinion that meiotic chromosome irregularities occur more frequently than in the parent species, though evidence of structural differentiation was less than in the Asiatic diploids.

Silow (43), while noting the evidence of structural differentiation in the sources cited above, concluded that there was so little evidence of structural irregularity that the differences in the main must be referred to a genetic basis. While this may yet prove to be true, it now seems to the writer an unwarranted conclusion from the data available. Marked evidence of structural differentiation is not to be expected in species with small chromosomes and few chiasmata. The typical configurations observed at meiosis in pure species of *Gossypium* as a whole are ring bivalents resulting from the formation of only one chiasma per chromosome arm. The replacement of a ring by a rod bivalent in interspecific hybrids therefore means that in such cases one entire arm of chromosome has probably failed to pair, and this failure is best interpreted as evidence of reduced structural homology. Since so few chiasmata are produced in cotton chromosomes, however, the amount of reduction in number of chiasmata which is possible within the limits of regular bivalent formation is very restricted, and the degree of chiasma reduction in interspecific hybrids does not provide a good quantitative estimate of the amount of structural differentiation. Metaphase pairing, in fact, approaches an "all-or-none" process, so that reduced number of chiasmata could be the result either of a high or of a low degree of structural differentiation. It is theoretically possible, for in-

stance, that some of the rod bivalents found in interspecific hybrids have only one pair of homologous arms, and, though this is not seriously considered as a likely situation, it is worth remembering that evidence from prophase studies or from genetic mapping of the chromosomes is at present quite inadequate to exclude it as a possibility.

Preferential Pairing in Allotetraploids Derived from Interfertile Species. It is clear that if two interfertile species have cryptic structural differences, the differentiation should be more readily observed in the allotetraploid obtained by colchicine treatment of their interspecific hybrid. Structural differentiation should here result in preferential pairing at meiosis, exhibited as a decrease in the number of polyvalent associations and an increase in the number of bivalent associations as compared with the corresponding associations in autotetraploids obtained from the parent species. Iyengar (*29*) presented the results of comparative meiotic studies in autotetraploid ***arboreum***, autotetraploid ***herbaceum*** and the allotetraploid, ***arboreum*** $\times$ ***herbaceum***, which prove conclusively that the parent species are structurally differentiated. Three strains of autotetraploid ***arboreum*** and two strains of autotetraploid ***herbaceum*** showed no significant differences in total number of metaphase associations per microsporocyte. Neither were significant differences found between three strains of allotetraploid ***arboreum*** $\times$ ***herbaceum***. But the allotetraploids on the average produced 19.8 ± 0.13 metaphase associations as compared with an average of 17.9 ± 0.20 in the autotetraploids, which is a highly significant difference. Examination of Iyengar's tables shows that the difference is in the main attributable to the production of more bivalents and fewer polyvalents in the allotetraploids, as expected if the parent diploid species are structurally differentiated.

In the amphidiploid cottons, ***hirsutum*** and ***barbadense***, it was found (5) that the F_1 hybrid after doubling with colchicine had an average of 15.2 quadrivalents at metaphase as compared with an average of 17.5 and 18.1 quadrivalents in ***hirsutum*** (doubled) and ***barbadense*** (doubled), respectively. The data as a whole are therefore consistent in showing that both diploid and amphidiploid species are structurally differentiated.

Now a little consideration leads to the conclusion that even the above comparative studies of auto- and allopolyploids yield only

a minimal estimate of the actual amount of structural differentiation of the parent species. Owing to the small number of chiasmata produced in cotton chromosomes, a large proportion of the metaphase chromosomes are represented as bivalents in both auto- and allopolyploids. Since structural differences are present, it is extremely likely that in the allopolyploids the majority of these bivalent associations are paired homologues from the same parent species. If this should be the case, structural differentiation may be much greater than the increased number of metaphase associations in allopolyploids might lead one to suppose. It is impossible to test this situation by cytological methods, as individual chromosomes can not be distinguished from each other with existing techniques. It is possible, however, to test it genetically with appropriately marked stocks, as will be indicated in a later section of this paper.

Comparative studies of polyploids involving Asiatic cottons (*arboreum* and *herbaceum*) and *anomalum* also yield significant information. There has never been any doubt that these species are structurally differentiated, since the F_1 hybrids at first metaphase produce an average of 10 to 12 bivalents out of a possible 13 bivalents, and quadrivalents, trivalents and univalents are common (48). Beasley (5), in studying similar material, concluded that four or more structural differences exist. Afzal *et al.* (2) found about the same number of bivalents as had previously been reported by Skovsted and other workers, but no higher associations were observed. They mentioned that Iyengar had found trivalents and quadrivalents in material identical with that used in their own studies, and also that chromatin bridges and fragments were present at first anaphase, and chromatin bridges at second anaphase. Of particular interest was their finding that the amount of structural differentiation in the interspecific hybrids (*arboreum* × *anomalum*) varied according to the strain of *arboreum* parent used. It seems to the writer that this could be interpreted as indirect evidence for the existence of small-scale structural differentiation, even within the *arboreum* species. However, it is probable that part of the differences observed could be attributed to the effect of different environments.

The gross structural differentiation, exhibited as polyvalents, univalents and chromatin bridges, which is evident in the above

studies is insufficient to explain the marked preferential pairing found in allopolyploids synthesized from hybrids of the parental species. It has been noted (52) that in allotriploids of the constitution A_2A_2D, the most common situation at first metaphase is that the *arboreum* chromosomes pair regularly as 13 bivalents, leaving the 13 chromosomes from the other parent unpaired. In the allotriploid obtained by crossing autotetraploid *arboreum* with *anomalum* it was expected, since the F_1 diploid *arboreum* × *anomalum* is partly fertile, that the situation in an autotriploid would be approached. (Skovsted (46) found six trivalents and additional higher polyvalents in an autotriploid Asiatic cotton). Actually an average of less than two trivalents per pollen mother cell was observed (54), which approaches the situation in the allotriploids examined earlier.

The allotetraploid *arboreum* × *anomalum* has been synthesized by several workers (3, 28, 54). There appears to be general agreement that it is considerably more fertile than the undoubled diploid hybrid, and that pairing is almost entirely between homologues from the same parent species, since it breeds true on selfing. The only published data on its meiotic pairing is given by Iyengar (28) who found 22.67 bivalents out of a possible 26. Preferential pairing to this extent is much greater than can be accounted for by the restricted amount of gross structural differentiation observed in the diploid hybrid, and the obvious conclusion is that the Asiatic (*arboreum* and *herbaceum*) and *anomalum* genomes are structurally differentiated throughout on a relatively minute scale, that meiotic pairing in the diploid hybrid therefore involves only partly homologous partners but does not entirely lead to the production of inviable gametes, and that pairing in the allotetraploid is almost entirely between homologous chromosomes, or at least that the gametes resulting from this type of pairing have a strong selective advantage.

Genetics and Breeding

Reduction of Crossing-over Between Linked Loci. Whatever the basis of interspecific differentiation in *Gossypium*, it is well established that interfertile species have many genetic loci with homologous series of alleles in common. This similarity in structure may be extended to the few linkage groups which are known.

The most striking example of the latter is the "Red-Cluster" linkage group (45) which is apparently common to the intersterile A and D genomes. In interfertile species the existence of common linkage groups offers a means of testing cryptic structural differentiation in the region between the linked loci. In interspecific backcrosses the linked genes should have a lower crossover value than expected on the basis of linkage tests conducted in intraspecific material, if structural differentiation reduces the chances of pairing within this region. In studying lint color inheritance in Asiatic cottons, Silow (44) found that lint color (Lc_1) was linked with leaf shape (L). Within *arboreum* the crossover value was 28.7%, but in *arboreum* × *herbaceum* backcrosses it was significantly lower (24.5%). When this reduction in crossover value is considered in relation to the cytological evidence of structural differentiation between these species, it would seem to have considerably more bearing on the speciation mechanism than Silow (43) attributed to it, though it is only fair to state that his opinion at the time was shared to some extent by the present writer.

Another linkage group which provides suggestive though not critical information is the "Green lint–Crinkle" linkage (17). Harland transferred the Crinkle gene from a White lint–Crinkle *barbadense* stock to a White lint–Normal *hirsutum* stock by successive backcrossing until all other visible *barbadense* characters had been eliminated and the Crinkle gene gave clear cut monofactorial segregation with its Normal allele in *hirsutum*. The transferred Crinkle was then tested against a *hirsutum* mutant, Green lint, and the two loci were found to be linked with 5% crossovers. One of the crossover types, Green lint–Crinkle, was maintained by selfing for several years and eventually used by Hutchinson (24) in further studies of the Crinkle allelomorph system. In an intra-*hirsutum* cross, in a backcross family of 305 plants, Hutchinson recorded 10% crossover values[1] between Green lint and Crinkle, *i.e.*, a looser linkage than determined by Harland in the same original material. A possible explanation of this discrepancy is that *barbadense* and *hirsutum* chromosomes

[1] An F_2 family examined at the same time gave approximately 5% crossovers, but this value was obtained only after transferring more than half of one of the crossover classes to a non-crossover class on the grounds of misclassification, and must therefore be regarded as suspect. Before adjustment, crossovers in F_2 and backcross both approximated 10%.

are structurally differentiated between the Green lint and Crinkle loci. If this is so, then on transferring Crinkle to a White lint–Normal *hirsutum* type, Harland brought in a section of *barbadense* chromosome which lies between these two loci. On crossing and backcrossing the transferred White lint–Crinkle to Green lint–Normal, crossing-over would be partially suppressed between these loci, but what crossovers occurred might reduce the length of the interstitial non-homologous segment and replace it by a homologous *hirsutum* segment. Consequently the crossover type, Green lint–Crinkle, would show a looser linkage in this region in subsequent test crosses with *hirsutum* stocks.

Skewed Backcross Ratios. The most striking feature in the progenies of interspecific hybrids is the failure to obtain clear cut genetic ratios in the segregations of genes which are known to be allelomorphic on the basis of intraspecific tests. Instead, the normal range of each phenotypic class is greatly extended and there is often considerable overlapping, with the result that the expected monofactorial segregations become blurred and cannot be classified. According to the multiple gene substitution theory of interspecific differentiation, the blurred segregations are attributed to the segregation of modifiers — admittedly a satisfactory explanation. Another feature which has received less emphasis is the skewed ratios obtained in the interspecific backcrosses. Although a common feature, the skewed backcross ratio is not so universal as the blurred F_2 segregation, but tends to be associated with specific genes or the chromosomes which carry them. They are most frequently found in transferring genes from one rather distantly related species to another, *e.g.*, in the transference of Asiatic Red from *arboreum* to *barbadense* (18), of several independent genes from *anomalum* to *arboreum* (42), and of Normal allele of Crinkle from *thurberi*, and petal color (anthocyanin) from *aridum*, to *barbadense* (22). In all these cases, when the backcross families are sufficiently large in size, there is a marked deficiency of the class containing the gene from the donor parent. The deficiency cannot be due to the effect of the introduced gene itself because with successive backcrosses the normal 1 : 1 ratio tends to be more closely approached. Neither can it be attributed to modifier segregation because the distinction between genetic classes is relatively clear cut. It has usually been noted that the

skewed ratios in the first and second backcrosses are accompanied by partial sterility and even aneuploidy, but these factors should have no differential effect on the ratios obtained unless the chromosome carrying the introduced gene is directly concerned. The writer prefers a more specific explanation along the following lines:

Partial sterility in early backcrosses implies that the chromosomes of the parental species are structurally differentiated, for which there is independent evidence in all the cases cited above. Deficiency of the introduced gene in backcrosses suggests that it is carried on an only partly homologous chromosome, or chromosome segment, the gametes containing which are at a selective disadvantage as compared with the gametes containing the corresponding chromosome from the recurrent parent. Further, the fact that in successive backcrosses the ratios tend to become "normal" suggests that crossing-over is occurring between the partly homologous chromosomes and hence, under the influence of selection, progressively removing "foreign" chromatin in the neighborhood of the introduced gene. The extent to which the gene becomes freed from its immediate neighbors should depend on the degree of structural differentiation between the chromosomes of donor and recurrent parent in this region.

If this explanation is acceptable when applied to species which are known to be structurally differentiated, then the occurrence of similar skewed ratios in crosses between species which are supposed to be structurally similar may be regarded as thought-provoking evidence. The best example is provided by the transference of the Crinkle gene from *barbadense* to *hirsutum* (13). The relevant data are analysed statistically below:

	Combined ratio Normal	Crinkle	X^2 (1:1)	X^2 Heterogeneity
1st backcross	166	98	17.5, P very low	5.5, P (2) = 0.05–0.10
2nd backcross	27	24	0.2, P = 0.50–0.70	4.9, P (5) = 0.30–0.50
3rd backcross	186	172	0.6, P = 0.30–0.50	0.7, P (2) = 0.70–0.80
4th backcross	9	10	0.05, P = 0.80–0.90	.....

It can be seen that there is a general tendency for the crinkle class to be deficient until the fourth backcross, but that the deficiency is statistically significant only in the first backcross where it amounts to as much as 25%. The writer has recently confirmed

this marked selection against an introduced Crinkle locus in a first backcross of *hirsutum* to *barbadense, i.e.*, in a reverse direction to Harland's transference, and also found similar though less striking evidence of selection against introduced genes at the leaf-shape locus. The interspecific hybrid *hirsutum* "Narrow" leaf (L^O) × *barbadense* "Intermediate" leaf (L^E) was backcrossed both to *hirsutum* "Broad" leaf (l) and to *barbadense* "Intermediate" leaf (L^E). In the backcross to *hirsutum* the L^E/l class was deficient, while in the backcross to *barbadense* the L^O/L^E class was deficient, which suggests that it is the leaf-shape chromosome from the donor parent which is at a selective disadvantage in either case.

Selective elimination of this nature has not been found in several other monofactorial differences which have been studied, but this, of course, does not prove that selective elimination is only of rare occurrence. It is hardly to be expected that, if it occurs, it would be detected in studies of single gene segregations except when the marked chromosome segment from the donor parent is at considerable selective disadvantage, *i.e.*, probably considerably differentiated in structure from its partner in the recurrent parent. Probably in the majority of cases the selective disadvantage would be so slight as to be obscured by chance fluctuations in the expected 1 : 1 ratios. It is, however, entirely possible that the effects of many small structural differences would be cumulative and hence detectable as a more rapid elimination of the donor parent genotype as a whole than would be expected on the basis of random recombination and segregation in successive backcrosses. An experiment to test this possibility is in progress at this Station at the present time.

The examples of skewed ratios which have been cited give clear evidence of selection against a chromosome segment introduced from the donor parent. The evidence may be regarded as critical, since the skewness disappears with continued backcrossing as expected or the skewness is reversed when the direction of backcrossing is reversed. In addition to these cases there are other suggestive examples in the literature which should be re-investigated. Harland (12), in a series of F_2s and backcrosses from the cross yellow corolla × white corolla, found a consistent excess of white segregates in both intraspecific and interspecific

crosses. The sole exception was in a selfed backcross (*hirsutum* white × *barbadense* yellow) × *barbadense* yellow where the skewness was reversed. The reason for the general excess of white segregates is unknown, but its reversal when the white gene is brought in from the donor parent is interesting and possibly significant. Hutchinson (23), in studies of corolla color in Asiatic cottons, found that a backcross of the type F_1 (*herbaceum* yellow × *arboreum* white) ♀ × *arboreum* white ♂ and its reciprocal gave an excess of white segregates, the deviation from the expected 1 : 1 ratio being greater when the F_1 was used as pollen parent. Intraspecific crosses involving the same genes gave good approximations to monofactorial ratios. This evidence cannot be considered critical because Hutchinson found that in one interspecific F_2 family the ratios obtained were apparently affected by the age of the seeds planted.

More general evidence consistent with cryptic structural differentiation is available in breeding data. In interspecific crosses between *hirsutum* and *barbadense* three or four backcrosses are sufficient to "complete" transference of a gene from donor to recurrent parent, *i.e.*, "complete" to the extent that the gene segregates normally, giving clear cut monofactorial ratios. At this stage of transference the backcross families are visibly so uniformly of the recurrent parent type, except for the introduced gene, that further selection is no longer effective (31):

"After the second or third backcross, the material is usually so near the backcross parent in appearance that large progenies possess little or no advantage. Small progenies become just as effective in eliminating any of the donor genotype which may remain . . . ".

Now if the visible differences between *hirsutum* and *barbadense* are attributable to multiple gene substitution, each allele from the donor parent, other than the one being consciously transferred, should on the basis of random recombination and segregation appear in the diminishing order of 50%, 25%, 12.5% *etc.* of the plants in the successive backcross families. Therefore, if interspecific differentiation depends on large numbers of genic differences it would seem inconceivable that the number of donor genes retained by chance after only three or four backcrosses would be so low as to produce no detectable effect in the backcross family.

Clearly, as has been emphasized (31), much of the donor parent genotype still remains in the third backcross, but this does not alter the fact that the visible approach towards the recurrent parent is much more rapid than the visible differences between the parents would lead one to suppose. In short, it may be reasonably concluded that there is a rather stringent selection against genes from the donor parent, and this conclusion is supported by the rarity of gene exchange between mixed crops of interfertile species (27). Unfortunately no specific tests have been set up to test the rates at which genetic markers are eliminated in interspecific backcrosses, but in 1947 the writer had the opportunity to see some breeding material at the Pee Dee Station, Florence, S. C., in which the possibility of introducing *barbadense* genes into *hirsutum* by mass backcrossing was being tested by Mr. W. H. Jenkins. According to this system, individual selection is not practised in the early backcross generations, but pollen from as many plants as possible is mixed and used in crossing to the recurrent parent. The original parents differed in three known monogenic characters, *viz.*, petal color and pollen color, which segregate sharply even in interspecific backcrosses, and petal spot, which is rather variable in expression. In the third backcross with no selection, therefore, one would expect that $3 \times 12.5\%$, *i.e.*, a little more than a third, of the plants should carry at least one of these introduced dominant genes. Actually these genes were found much more rarely than expected, and if the material is in any way representative of the usual situation in interspecific backcrossing, there can be no doubt that there is a strong selection against specific introduced genes or the chromosome segments which carry them. According to the multiple gene substitution theory, the only mechanism to account for differential elimination would be selection against gametes of zygotes which possess a "modifier complex" different from that of the recurrent parent, but it is difficult to see why any particular gene from the donor parent should have a markedly smaller chance of inclusion in the favored complex. Selection on this basis would be a long slow process and surely not detectable in monogenic class differences. A more logical explanation is that the chromosome carrying the introduced gene is at a selective disadvantage, or more precisely that the gene is carried on a differential chromo-

some segment. On this alternative basis it would be expected that in the interspecific hybrid both the non-crossover chromatids, carrying a differential segment from the donor parent, and crossover chromatids, carrying the consequent deficiencies and duplications, would be at a selective disadvantage as compared with non-crossover chromatids of the recurrent parent type.

Block Transference. As suggested in the previous section, structural differentiation would be expected to lead to a rapid elimination of donor parent genes in backcrosses, actually far more rapidly than a mechanism based on modifier differences. Conversely, conscious artificial selection of donor parent genes would be expected to maintain, against the forces of natural selection, whatever small blocks of non-homologous chromosome segments happened to be associated with the gene selected. In other words, backcrossing involving structurally differentiated species would imply introduction of blocks of linked characters rather than transference of individual genes, as expected by the multiple gene substitution hypothesis. These implications may have an important bearing on Harland's statement (19) that "in no case where a multiple allele series has been established is a dominant character common to both species", and further that "cases of identity (apparent or real) are practically confined to recessive genes only". There can be no doubt that when homologous series of alleles are found to be common to *hirsutum* and *barbadense*, the relative frequencies of the alleles are different in the two species (15, 19, 20). A similar situation is found in comparative studies (42) of the Asiatic cultivated species. But the evidence that Normal, or "wild type", dominants corresponding to certain mutants are different in different species is much less easily demonstrated. Hutchinson (24) did not find evidence for as many Normal alleles of Crinkle as Harland had formerly supposed, and with regard to the leaf-shape alleles, Stephens (55) pointed out that while tests of allelomorphism are relatively easy, tests of identity furnish a much more difficult proposition. In the case of the leafshape alleles a more critical analysis was possible. It was found (55) that three leaf-shape "alleles" occurring in *G. barbadense* var. *darwinii*, *G. hirsutum* var. *mariegalante* and *G. hirsutum* (Upland), respectively, were associated with phenotypically very similar leaf shapes. On transference to

a common Upland background, however, their leaf indices were found to be significantly different statistically, and the difference was not reduced by further backcrossing. It might therefore have been concluded that different dominant alleles were characteristic of the three subspecies. This conclusion was not acceptable because it did not agree with the results of more critical developmental studies. It was found (53, 56) that the shape of the climax leaf in *Gossypium* is mainly dependent on three developmental processes: (*a*) the rate at which a juvenile undissected leaf is converted into a lobed adult leaf, which may be represented graphically as a "developmental track"; (*b*) the rate at which development proceeds along each track; and (*c*) the distance progressed along each track during the vegetative period of the plant's life history. Of these three processes only (*a*) is determined by the leaf-shape allele, while (*b*) and (*c*) depend predominantly on the flowering habit of the plant. It is therefore of considerable significance that process (*a*) was found to be almost identical for the three leaf-shape "alleles" under study, and the phenotypic difference between them could be wholly ascribed to processes (*b*) and (*c*). The most probable explanation is that the leaf shapes in the three subspecies are determined by identical leaf-shape genes, and that the differences between the transferred types were due to associated blocks of linked characters brought into the recurrent parent during backcrossing. Differences of this type can be analyzed only in certain favorable cases, and they show clearly that generalizations based on comparatively superficial phenotypic expressions may be rather misleading. It will be noted that the suggestion that the leaf-shape locus may be associated with a differential segment receives some support from the skewed ratios obtained in connection with segregation of this locus which have been cited earlier.

The idea that backcrossing one species to another, with selection of desirable characters from the donor parent, often results in the unintentional introduction of undesirable blocks of characters as well, finds some support in breeding experience. Unfortunately the data which could provide critical information on the nature of interspecific differentiation often go unrecorded and can be gleaned only in verbal discussions with plant breeders. Nevertheless, the considered opinion of people who have unrivalled

opportunities for studying the segregation of characters in large populations must carry some weight. The synthetic amphidiploid, *arboreum* × *thurberi* (4), was successfully crossed and backcrossed to Upland (*hirsutum*) cotton. Analysis of fiber properties of the early backcross progenies showed that some of the segregates had greatly increased fiber strength (6) which could be attributed to certain fiber properties (low X-ray angle and narrow perimeter) inherited from the *thurberi* parent. Further work carried out independently in Texas and in North Carolina has shown that this property can be maintained through successive backcrosses, but there is an apparently strong negative correlation between strength and high yielding capacity (39) which has not yet been broken. In some lines plants with strong fibers tend to be less vigorous and to carry small abnormally shaped bolls; in others boll production is prolific, but the individual bolls are small and lint production low. In still other lines strength seems to be associated with partial sterility. Now physiological studies of the factors influencing fiber strength in *hirsutum* cottons (6) have shown that this character is affected in a highly complex manner by the general water relations of the plant, and that the factors which favor high strength are likely to be unfavorable to high yield. One might therefore suppose that the negative correlation between strength and productivity observed in the interspecific backcrosses is a necessary physiological consequence, and not attributable to genetic linkage. But in this case one would expect that the inheritance of high strength would be exceedingly complex from the genetic point of view, and that as a consequence it would be difficult to maintain strength through successive backcrosses. Actually a reverse situation is found, as strength is maintained and the proportion of strong fibered types suggests that rather few genes are involved. Physiological complexity and apparent genetic simplicity are not irreconcilable if the strength characters are carried on comparatively few differential chromosome segments which have been introduced by backcrossing into only partly homologous *hirsutum* chromosomes. In this case one would expect that the crossover chromatids, carrying differential segments, either might contain deleterious *thurberi* genes in blocks tightly linked with the strength genes, or might be deficient as a result of the initial crossovers. This interpretation finds some

support in the fact that the chromosomes of *thurberi* and *hirsutum* are known to be structurally differentiated, and, further, in the observation (Dr. T. Kerr, personal communication) that when the selection pressure for strength is relaxed, strong fiber types are rapidly lost from the breeding material.

Block transference of this type is not limited to crosses between species, like the above, whose respective chromosome sets show gross structural differentiation. Parallel cases are found in crosses between *hirsutum* and *barbadense*. Knight (30) successfully transferred two dominant genes (B_2 and B_3) for Black-arm resistance from *G. hirsutum* var. *punctatum* Hutchinson to *G. barbadense*. The expected bifactorial segregation (3 resistant : 1 susceptible) in successive backcross generations was violently skewed (approximately 2 resistant : 1 susceptible), which was shown to be due to linkage between B_2 and B_3. Pooling the results of seven backcrosses, Knight arrived at a crossover value of 37.2%, but this value showed an overall trend, *i.e.*, it tended to increase with successive backcrossing. Inspection of Knight's data (Table 9) shows that the average crossover value calculated from the total of the first four backcrosses was 32.4%. In the fifth backcross there was an "apparent sudden loss of linkage", and the crossover value jumped to 48.5%. The average crossover value calculated from the total of fifth, sixth and seventh backcrosses was 41.3%. It seems likely that whatever causes were responsible for the increased chiasma frequency in the fifth backcross parents, they increased the chances of further crossing over in this region in subsequent generations. This is consistent with the hypothesis that the *punctatum* and *barbadense* parents had structurally differentiated chromosomes in the B_2 and B_3 region, and that the unexpectedly high crossover frequency in the fifth backcross parents resulted in the permanent replacement of a differential segment by one of the recurrent parent type.

In a more general way, evidence consistent with block transference is provided by the long continued attempts of cotton breeders to transfer desirable fiber characters from Sea Island (*barbadense*) to Upland (*hirsutum*) cottons. Of the extensive work carried on at Florence, S. C., Mr. W. H. Jenkins kindly informs me that it is "very difficult to carry over desirable characters of the Sea Island without carrying also undesirable characters such

as low yield, low lint percent, small bolls, *etc.* Only by intensive selection at every stage has it been possible to carry desirable fiber characters, and it has taken long hard work to develop relatively few strains".

Blurred Monofactorial Segregations and Depressed Vigor in Selfed Hybrid Progenies. The multiple gene substitution hypothesis received its strongest support from the behavior of selfed progenies of interspecific hybrids. According to the hypothesis, multiple segregation of the genes comprising the "modifier complexes" of each parent species leads to a breakdown in their respective genetic architectures which have been built up by natural selection, with the result that most of the segregates are inferior to either parent. Similarly, effective genic action in each species is adjusted to the internal genetic environment built up by natural selection so that segregation in F_2 disrupts the appropriate *milieu*, and genic expression becomes highly variable.

There can be no doubt that this hypothesis furnished a satisfactory interpretation of these phenomena, but here it must be pointed out that it is not the only possible interpretation. Exactly the same behavior would be expected if the parent species were structurally differentiated in the cytological sense. On the latter alternative interpretation we should replace the rather vague concept of a genetic architecture built up of "constellations of modifiers", by a more precise cytological architecture depending on (*a*) the linear order and (*b*) the nature of the genetic loci throughout the chromosome set. In different species neither (*a*) nor (*b*) would be identical, though in interfertile species a rather close resemblance might be inferred. The interspecific hybrid would contain a complete genome from each parent, and the resemblance between corresponding but not strictly homologous chromosomes might be expected to lead to regular bivalent pairing. In *Gossypium* the low chiasma frequency results in a proportion of rod bivalents, *i.e.*, bivalents in which only two out of four chromatids are involved in a crossover. From such bivalents the chromatids delivered to the gametes would be of three types, (*a*) and (*b*), non-crossover types identical with the respective parental chromosomes, and (*c*), crossover types which, owing to structural differentiation, would carry deficiencies and their reciprocal duplications. Depending on the degree of structural differentiation be-

tween the parent species, gametes or zygotes carrying crossover chromatids would be at a selective disadvantage, and the most vigorous F_2 segregates would be those carrying the most non-crossover chromatids. That is, they would be phenotypically like either parent or like the F_1. Further crossing-over in later generations would tend to eliminate the F_1 type and to increase the number of parental type segregates. This is, of course, exactly what is found when interfertile species of cotton are grown together in mixed cultivation. The majority of the plants can be classified as belonging to one species or the other, the only exceptions being those which resemble the F_1. In experimental culture, segregates carrying crossover chromatids would be duplicated in respect to some segments and deficient in others, though in the zygote a considerable amount of mutual masking might be expected. It is possible that the "plus and minus modifiers" of the gene substitution hypothesis might be accounted for by duplications and deficiencies, respectively. In backcrosses all deficiencies would be masked by the complete chromosome set of the recurrent parent, and hence "modifier disturbances" should be greatly reduced. This is actually the case, and in fact many gene segregations which cannot be scored in F_2 give clear-cut segregations in the first backcross. Continued backcrossing should in the absence of conscious counter selection result in a rather rapid replacement of differential segments by those of the recurrent parent type.

Pseudo-alleles. The evidence so far presented has shown that various cytological, genetic and breeding phenomena in interfertile species are consistent with the hypothesis that the species are structurally differentiated. The problem may also be approached in another way, *viz.*, in a search for evidence of the mechanism by which specific chromosome segments are becoming structurally differentiated. In order to consider this aspect it is necessary to review briefly some developments in general genetic theory.

The great majority of mutant alleles are at least partly recessive, *i.e.*, the heterozygote between wild type and mutant allele shows either complete dominance or intermediacy. Comparatively rarely, cases occur where the heterozygote shows a new character different from either parent—the "alleles" appear to have a

complementary effect. This situation is found in certain long multiple allelic series, *e.g.*, in the *A* and *R* loci in maize (9, 49) and the elytra pattern series in *Harmonia* (61). Certain cases of complementary "allelism" have recently been shown not to be due to allelism at all, but to the interaction of non-homologous but closely adjacent loci, *e.g.*, the Yellow-green/Pale Yellow series in maize (34), the Scute series (41) and Star/Asteroid (32) in *Drosophila* and the anthocyanin series (65) in cotton. Since the pseudo-alleles, in spite of their complementary interaction, appear to retain a basic similarity in function, it appears likely that they have arisen as repeats of an originally single locus (32, 58) in a manner very similar to the origin of Bar and Double-bar (7) in *Drosophila*. Such an interpretation is not new, as both Haldane (11) and Muller (35) had suggested earlier that duplication offers a possibility for the development of new loci. The significant feature is that pseudo-alleles resemble independent genes and differ from true alleles in the fact that they produce a complementary effect in the heterozygous condition. In short, they behave as would be expected if they represented the first stage in the development of cytologically and biochemically new loci. If such should be the case, the production of pseudo-alleles would represent a highly important evolutionary step, since it would afford a means whereby structural differentiation and the development of new characters could be accomplished simultaneously. As such it might have a greater evolutionary significance than "point mutation", as the latter in itself provides no mechanism for the origin of new loci and in many cases (62) leads to the inactivation of loci already present.

Considering now the situation in cultivated cottons, it is to be noted that out of 17 independent gene-controlled characters in Asiatic cottons listed by Hutchinson and Silow (26), anthocyanin pigmentation and possibly also Crumpled are controlled by "alleles" which give complementary effects (59, 65). In the cultivated amphidiploid cottons, out of 13 characters studied (excluding duplicates), Crinkle and Corky are both controlled by complementary "alleles" (24, 57, 59). Of these four complementary "allelic" series, one (the anthocyanin series) is known definitely to be pseudo-allelic (65), and it is suspected that the other three may have a similar basis. It may well be, therefore, that about

10 per cent of the known loci in cotton are in the process of forming new daughter loci, *i.e.*, the first step in structural differentiation.

A somewhat similar line of evidence for small scale structural differentiation is provided by complementary genes. In cotton, as pointed out by Silow (45), it is not easy to distinguish between duplicates (giving 15 : 1 F_2 ratios) and complementaries (giving 9 : 7 ratios). The difficulty, however, goes deeper than the dominance modification with which he was concerned. It is possible to trace the successive steps by which true duplicate genes like the chlorophyll deficiency genes in amphidiploid cotton (14, 16) may become converted into complementary genes like the corolla color (42), pollen color (26), glabrous lintless and hairy lintless complementaries (25). The clue to the relationship was provided by Silow's discovery (45) of a duplicated linkage group in the amphidiploid cottons. Anthocyanin (R_1) and Cluster (Cl_1) form a linkage group in the D sub-genome, and Anthocyanin (R_2) and Short branch (Cl_2) form a parallel group in the A sub-genome of the amphidiploid (AD) cottons. Genes and their mutants at the R_1 locus are similar to, but not identical with, those at the R_2 locus; Cluster and Short branch would probably be indistinguishable in otherwise isogenic stocks. The facts that the linkage groups correspond, that they are carried in different sub-genomes, and that the R_1 and R_2 loci have been shown to be homologous with loci carried by the corresponding diploid relatives (18, 22), remove any doubt that these loci have evolved from common ancestral loci. With amphidiploidy, both loci were duplicated. But they are no longer duplicates. Genes at the R_1 and R_2 loci, though similar, give complementary effects in combination; for example, the interaction R_2 Ghost Spot with R_1 Spotless to give Red Spot (42). Cluster and Short branch give complementary Normal branch in combination (45). In short, these complementary "duplicates" bear exactly the same relationship to true duplicates as pseudo-alleles bear to true alleles, *i.e.*, they have diverged in function.

Further evidence of this relationship is provided by Silow's comparative studies (42) of anthocyanin pigmentation in *arboreum* and *anomalum*. In *arboreum* the production of a red spot in the petals depends on the interaction of two pseudo-alleles, Spotless and Ghost Spot (65). In *anomalum* a similar interaction is

found, but the Spotless and Ghost Spot loci are carried in different chromosomes. Silow found that *arboreum* Ghost Spot and *anomalum* Ghost Spot were either identical or allelomorphic, while *anomalum* Spotless was a duplicate of *arboreum* Spotless. He assumed that a recessive allele was carried by *arboreum* at a locus homologous with *anomalum* Spotless, so that according to his interpretation the constitution of a red spot in *arboreum* and *anomalum* would be (R^GR^S); r^O and (R^Gr^O); R^S, respectively. There is, however, no good reason to assume this structural homology. It is quite possible that the *anomalum* Spotless locus is carried in a translocated segment, which would be more in accordance with the fact that backcross ratios were skewed, and also with the independent evidence that occasional quadrivalent associations are found at meiosis in the interspecific hybrid. In this case the r^O symbols in the preceding formulae could be replaced by "blanks". Of particular interest was Silow's finding that the *anomalum* Spotless gene, though it resembled *arboreum* Spotless in giving a complementary interaction with Ghost Spot, was not identical with it. In *anomalum* Spotless a small amount of anthocyanin is present throughout the petal which interacts with the yellow pigment to give a "gold petal" or bronzing effect. In *arboreum* Spotless no anthocyanin is produced until the flower fades. Here again there is the suggestion that "duplicates" have undergone a slight divergence in function, and further that duplication and complementary interaction may be intimately related.

With regard to corolla color, Silow (42) found that a full yellow color depends on Y genes at three independent loci being present simultaneously. When any one of the Y genes is replaced by a recessive, Y^P, the corolla is pale yellow. Any two different pale yellow types are therefore complementary with each other giving a full yellow hybrid. On selfing, a ratio of 9 full yellow : 7 pale yellow is obtained in F_2, but since the different pale yellow types are usually distinguishable from each other, the ratio can be further broken down into 9 full yellow : 3 pale yellow (parent type A) : 3 pale yellow (parent type B) : 1 near-white (double recessive). The pale yellows can therefore be considered either as complementaries or as independent genes, according to the accuracy of scoring. However, as Silow has indicated, there is some reason

to believe that the three *Y* loci were originally duplicates. This receives some support from the chemical evidence (37) that full yellow petals contain a mixture of three anthoxanthin pigments, gossypetin, quercetin and herbacetin. Quercetin and herbacetin are isomeric in chemical structure, and gossypetin may be regarded as an internally compensated "hybrid" of the other two. The writer's preliminary and unpublished studies of the three pale yellow complementaries suggest that they differ from full yellows and from each other only in the relative proportions of these three pigments. It may therefore be inferred that the nature of the gene-controlled processes associated with the three complementary loci are closely related, *i.e.*, probably had a common origin. Do these complementaries represent a transition between duplicates and independent genes, since they share some of the characteristics of both?

The glabrous lintless complementaries give a normal hairy linted phenotype in combination, and the F_2 segregates in the ratio 9 hairy linted: 7 glabrous lintless. The hairy lintless complementaries are similarly related. The glabrous lintless complementary pair is distinguishable from the hairy lintless pair by the fact that the former removes all hairs from the vegetative surface of the plant, while the latter removes only the seed hairs. Crosses between glabrous lintless and hairy lintless therefore give independent segregations in F_2 (9 hairy linted : 3 hairy lintless : 4 glabrous lintless). Were it not for their differential action on vegetative hairs, all four loci would be regarded as complementaries, any two of which on crossing together would give 9 linted : 7 lintless in F_2. Is it possible that the difference between the lintless complementary pairs represents the final stage in a transition from complementary to independent genes?

The data which have been considered may be summarized as follows: There is a certain amount of genetic and chemical evidence that in *Gossypium* a mechanism exists by which small scale structural differences (repeats) and the production of qualitatively new loci can occur simultaneously. Studies of complementary genes suggest that they may possibly be interpreted as transition stages between duplicates and independent genes. The importance of this mechanism in the speciation of the genus has yet to be

assessed, but it must at least contribute to the structural differentiation which has been found by independent cytogenetical investigations.

METHODS OF TESTING FOR CRYPTIC STRUCTURAL DIFFERENTIATION

As pointed out earlier, the evidence reviewed in this paper is almost entirely incidental and not obtained as a result of experiments set up specifically to test the existence of structural differentiation. From the taxonomic point of view it is probably of little significance whether species distinctions are attributed to multiple gene substitution or to cryptic structural differences, but from the economic standpoint it is an issue of prime importance. Upon it depends the degree to which a plant breeder can hope to transfer a character of potential economic value from one species to another. If species differences are mainly attributable to multiple gene substitution, the problem is chiefly a matter of growing large enough progenies to secure the required combination. Alternatively, if species are structurally differentiated, there is no way available at present to open the "surprise parcels" of good and bad characters which may be transferred with every differential segment. It is therefore important to devise more precise methods of testing the nature and extent of interspecific differentiation.

The first method is available to all cotton breeders, since it consists merely of recording the rates at which various marker genes from the donor parent are lost in successive backcrosses. Mass backcrossing and reciprocal backcrossing afford particularly favorable material for this type of investigation.

The second method is based on the testing of linkages. Few linkage groups are known in cotton, but those which are known are common to two or more interfertile species. By comparative studies of crossover percentages between the same linked loci in intraspecific and interspecific backcrosses it would be possible to determine whether the apparent linkage is closer in the latter case, as expected if the chromosomes of the parent species are structurally differentiated within the limits of the tested region.

The third method depends on the preferential pairing expected in allopolyploids synthesized from fertile interspecific hybrids. As shown earlier, comparison of meiotic associations in allo- and

autotetraploids provides a qualitative test of structural differentiation, but gives a very poor quantitative estimate. By using stocks from two interfertile species which carry the same genetic markers, allo- and autotetraploids can be synthesized which have the same genetic constitution in respect to the marker, *viz.*, *GG gg*. They will differ in the fact that in the allotetraploid the chromosomes carrying *G* will be from one species and those carrying *g* from the other, while in the autotetraploid all four chromosomes will be from one species. Preferential pairing will be indicated if the proportion of *gg* gametes produced by the allotetraploid is significantly less than the proportion produced by the autotetraploid, and the "preferential index" obtained should furnish a quantitative estimate of structural differentiation in the chromosome carrying the marker locus. It should be noted that the test is comparative and independent of agreement with the theoretical polyploid segregation. Of the various cytological factors (33) which influence the latter, *viz.*, mode of pairing, proportion of quadrivalents, chiasmata frequency, position of the locus relative to the centromere, the last named will be identical in both types of tetraploid, and the other three will be determined primarily by differential affinity which is the object of the test. The partial sterility of the tetraploids, and the consequent difficulty of obtaining progenies of adequate size, can be avoided to a great extent by using recessive (*gggg*) *hirsutum* as the tester stock in place of a multiple recessive autotetraploid. The sterile hybrid, *hirsutum* × tetraploid *arboreum*, can be obtained easily (52) and is very vigorous.

SUMMARY AND CONCLUSIONS

The evidence presented in this review shows clearly that multiple gene substitution, as suggested by Harland, is not sufficient to explain the cytological, genetic and breeding phenomena encountered in critical studies of fertile interspecific hybrids and their progenies in *Gossypium*. Studies of F_1 hybrids and of allotetraploids synthesized from them have provided evidence of cytologically detectable structural differentiation. Since few chiasmata are formed at meiosis in cotton species in general, it is clear that only relatively gross structural differences are likely to be observed. Such studies, therefore, do not provide a reliable quan-

titative estimate of the total amount of structural differences present. Recent evidence from studies of synthetic amphidiploids casts doubt on the validity of "normal" chromosome pairing and hybrid fertility as indices of structural homology. The following genetic and breeding phenomena in hybrid progenies are more consistent with cryptic structural differentiation than multiple gene substitution: (*a*) reduced crossing over; (*b*) skewed backcross ratios and rapid elimination of "foreign" genes; (*c*) block transference of characters resulting from conscious selection of only one gene. The postulated multiple gene substitution mechanism received chief support from the depressed vigor and blurred monogenic segregations found in selfed progenies; both these conditions, however, are equally well explained by crossing-over between similar but only partially homologous chromosomes. The relatively high proportion of "alleles" and "duplicates" which give unexpected complementary interactions suggests that a mechanism whereby biochemically new loci and small scale structural differences may be developed simultaneously is operating in cotton.

The general conclusion to be reached from these studies is that both multiple gene substitution and cryptic structural differentiation have determined the building of species from the earliest stages onward. The importance of the latter mechanism has yet to be assessed, but almost certainly has been greatly underestimated in the past. It is important now to devise critical tests, some of which have been suggested in this review, in order to obtain more quantitative evidence. The results are not likely to invalidate the inferences of taxonomic significance which have been drawn from the multiple gene substitution hypothesis, but they may have an important bearing on evolutionary mechanisms and breeding technique. The success which the breeder can expect in transferring potentially valuable characters from one species to another will be inversely proportional to the frequency of small scale structural differences in his breeding material.

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ISOTOPES AS TRACERS IN PLANTS

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INTRODUCTION

Isotopes afford an extremely useful means for following the absorption and transport of materials in plants and for elucidating the metabolic reactions of plants. The potentialities of the tracer method were early recognized by Hevesy who with Paneth in 1913 (72) used radiolead* as an indicator to determine the solubilities of lead chromate and lead sulfide, and in 1923 (63) followed the absorption and translocation of radiolead in plants. He applied the tracer technique in the same manner that it is used today (63), described its advantages and suggested how it could be employed.

The early limitation of the tracer method to naturally occurring radioisotopes was removed by the discovery of artificially induced radioactivity by Joliot and Curie (83), and the use of stable isotopes as tracers was made possible by Urey's methods (146, 150) of separating D, N^{15}, C^{13}, O^{18} and S^{34} in usable quantities. Supplementary developments that have been particularly helpful are the production of uranium piles (nuclear chain reactors), cyclotrons and other particle accelerators, simplified mass spectrometers for measurement of stable isotopes, and commercial Geiger-Müller counters and electrometers for measuring radioactivity. Since the release of radioisotopes by the Atomic Energy Commission (see Science **103**: 697-705. 1946; and the catalog and price list of radioisotopes distributed by the Isotopes Branch, U. S. Atomic Energy Commission, Oak Ridge, Tenn., for lists of radioisotopes and the form in which they are supplied), it has become unnecessary to maintain elaborate high voltage particle accelerators for most tracer work, as neutron bombardment in the uranium pile produces satisfactory radioisotopes of almost all elements of biological interest. Deuterium and O^{18} are also available through the facilities of the Atomic Energy Commission. N^{15} and C^{13} may be purchased from Eastman Kodak Company; individual institutions no longer need maintain thermal diffusion columns for the concentration of C^{13}.

* The term radiolead, radiopotassium, *etc.*, will be employed to designate radioactive elements as suggested by Joliot and Curie (83).

Prior to the war the elaborate instrumentation necessary to produce and measure isotopes greatly limited their use. Now these obstacles have been removed, and with a modest investment a laboratory can be equipped for work with isotopic tracers. The use of isotopes is expanding enormously, and this trend will doubtless continue.

In explanation it should be stated that this review makes no claim to be an exhaustive survey of the literature. Some journals from the war years have not been available; certain review articles which describe original papers in no greater detail than the current review have been omitted; the literature on animals, yeasts, molds and bacteria has not been covered; and unquestionably some papers which should logically be included have been overlooked. This paper does not purport to describe methods in any detail. Kamen's (85) and Hevesy's (68*b*) recent books, the compilation of papers edited by Wilson, Nier and Reimann (157) and the University of Wisconsin Symposium on Isotopes (150) serve as excellent manuals for those wishing to employ isotopes as tracers. The following are useful references on the general application of isotopes: (28, 56, 59, 60, 65, 67, 68*a*, 73, 84, 87, 90, 92, 97, 102, 119, 120, 124, 127, 128, 138, 139, 140, 152, 160).

THE METHOD AND ITS MODE OF APPLICATION

The isotopic tracer method is basically very simple. A compound is labeled with an isotope, added to a chemical reaction mixture or to a biological system and allowed to react. After a suitable period the reaction is stopped, and reaction products are isolated and analyzed for their content of the isotope in question. The reaction mechanism is then interpreted on the basis of the distribution of the isotope in the products isolated. Thus the basic elements of the quantitative tracer method are synthesis, reaction, isolation, analysis and interpretation. As a variation of this scheme, the qualitative distribution of radioisotopes can often be followed in intact organisms without resorting to isolation procedures.

Synthesis usually involves no particular difficulty when one is interested in the uptake, transport or metabolism of inorganic ions, but is frequently a major problem when organic compounds are being traced. C^{14} was supplied initially as barium carbonate only, but it is now possible to obtain it commercially from Tracerlab,

Boston, through the facilities of the Atomic Energy Commission, incorporated into barium carbide, acetylene, sodium cyanide, methanol, methyl iodide and carboxyl-labeled sodium acetate and ethyl acetate. The Atomic Energy Commission will also furnish O^{18} as H_2O^{18} and deuterium as either D_2 or D_2O; deuterium makes an excellent label for carbon chains when incorporated into stable linkages. Methyl iodide, NaCN and $BaCO_3$ enriched with C^{13} are now available from Eastman Kodak Company for those wishing to use the stable carbon isotope; this company also supplies $N^{15}H_4NO_3$ and N^{15}-enriched potassium phthalimide. Although starting materials for syntheses are limited, efficient methods for the production of many labeled compounds have been worked out. Usually the syntheses have involved well known methods adapted to small scale procedures and modified to improve yields relative to the isotopically enriched starting material. A number of these syntheses have been published (96, 102, 124 section V, 150 page 161).

Biosynthesis may often be useful for the production of complex labeled compounds, compounds such as starch (95), glucose and other sugars (122) which cannot be synthesized by chemical means, and the naturally occurring form of optically active compounds. In addition biosynthesis of certain simple compounds can compete directly with chemical syntheses on a basis of yield and convenience. A notable example is the production of certain organic acids by microorganisms (86); fermentation mechanisms for the production of these acids have been worked out in detail so that the location of a carbon label is well defined. Well-defined biosyntheses also may be accomplished with isolated enzyme preparations. The fermentation of labeled sugar and isolation of the products will also yield labeled compounds. Higher plants synthesize a tremendous variety of compounds, and isotopic CO_2 supplied to plants in closed containers is reduced by photosynthesis and eventually appears in these compounds to label their carbon atoms. The chief disadvantage of such syntheses is that the isotopic carbon may be distributed in the compounds in an unknown fashion. After biosynthesis the labeled material must be isolated, purified and its identity established before use.

Although our considerations have centered around the incorporation of isotopic carbon into compounds, other elements can

also be added as labels by biosynthesis, *e.g.*, P^{32} in phosphorylated compounds and N^{15} in amino acids. The most convenient agents for biosynthesis of N^{15}-labeled amino acids are microorganisms, such as *Escherichia coli, Proteus vulgaris, Azotobacter vinelandii, Torula utilis* and *Saccharomyces cerevisiae,* which can utilize ammonia as their sole source of nitrogen. Their high protein content decreases the interference by extraneous material in isolation procedures, and by starting with small inocula very little dilution of the N^{15} supplied is experienced.

Following synthesis, the isotopically labeled compound is allowed to react in the system under investigation. The experimental conditions at this step will vary widely—the compound may be administered through the root system, through cut stems or petioles, infiltrated into intact or excised leaves, injected, given as a gas or vapor, applied in a paste or solution, or added to tissue slices, minces, homogenates or cell-free solutions. After a suitable time is allowed for reaction, the system is inactivated and products are isolated. Again the methods employed will be diverse and will be dictated by the nature of the experiment. Although it is impossible to generalize on the subject of isolation of products, it should be emphasized that the compounds isolated must be carefully purified. Constancy of the specific activity of a compound distributed between sets of solvents is useful in establishing its purity. Isotopes can be measured in such great dilution that a small trace of impurity is detected readily and may confuse the interpretation of results. The isolated products are analyzed for their isotopic label, and both their total isotope content and their specific activity (atom per cent excess for stable isotopes) are usually reported. Semi-quantitative results may be achieved by less laborious procedures, such as the radioautography (49) or counting of strips (88) from filter paper partition chromatograms. These data permit the investigator to interpret the metabolic reactions undergone by the labeled compound administered.

COMPARISON OF STABLE AND RADIOACTIVE ISOTOPES

Both stable isotopes, concentrated above their natural abundance, and radioactive isotopes may be employed as tracers. The choice between the two is often plain; for example, stable N^{15} and O^{18} are chosen because the radioisotopes of these elements have

very short half-lives. On the other hand, the radioactive isotopes of phosphorus and sodium, P^{32} and Na^{24}, are indicated, as only a single stable isotope of each element exists. The choice between H^2 and H^3, C^{13} and C^{14}, S^{34} and S^{35} may not be so clear but will be governed by such considerations as the analytical equipment available to the investigator, the dilution to be experienced, whether there is a desire to stock a compound requiring a time-consuming synthesis (stable S^{34} *vs.* radioactive S^{35}), and the necessity for following the material in the intact plant.

The advantages of the stable tracers can be listed as their indefinite stability, their lack of radiation hazard and their adaptability to accurate quantitative work. The limited number of concentrated stable isotopes available is offset by the fact that the ones available are of great biological importance; if others were seriously needed they could unquestionably be concentrated.

Radioelements can be formed of almost any element, and although it does not necessarily follow that they will all be suitable as tracers, many of them serve as excellent labels. The radioelements have the advantage that they can be measured at tremendous dilutions; dilutions much greater than a million fold are often practical, whereas with the stable isotopes one is normally limited to a dilution of about a thousand fold. Radioisotopes are generally easier to measure than are the stable isotopes, and the equipment required is relatively simple and inexpensive. It is impossible to detect the stable isotopes except by destroying the test plant and analyzing with a mass spectrometer (there are alternative methods for measuring deuterium, such as the falling drop and refractive index methods), whereas radioactive isotopes which emit sufficiently energetic radiations can be measured by placing a Geiger-Müller counter tube or an ionization chamber near intact parts of the plant. Radioautographs (48) will also establish the distribution of radioisotopes in the plant.

It should be noted that quantitative methods are inherent in the technique of using stable isotopes. Similar rigorous criteria, if regularly applied to the use of the radioactive tracers, would greatly enhance the value of much of the tracer work conducted. Qualitative studies are of course extremely useful, but the magic of the word "isotope" and the ease and speed with which experiments can be conducted with radioisotopes have tempted us to

accept observations without critical appraisal. Too often a labeled compound has been administered, and all radioactivity subsequently measured has been attributed to the intact molecule of the administered compound, whereas a radioisotope is actually detected whether it be in the original compound or any of a multitude of others formed from it as metabolic products.

Undesirable properties of radioisotopes include their radiation hazards to personnel and the ill-defined but sometimes deleterious effect of their radiations on the tissue being tested. Since it was possible safely to handle multicurie amounts of radioactive materials in the production of U^{235} and plutonium during the war, it is obvious that with suitable precautions tracer quantities of radioisotopes can be used without undue hazard. The dangers must not be minimized, however, and each laboratory should establish strict rules for handling and disposal of radioactive materials. (The Isotopes Branch, U. S. Atomic Energy Commission, P. O. Box E, Oak Ridge, Tennessee, will supply on request its circular B–1, "General Rules and Procedures Concerning Radioactive Hazards"). The worker's chief protections are shielding, distance, limited time of exposure, avoidance of direct contact with radioisotopes, scrupulous cleanliness and vigilant monitoring of work areas.

It has been argued (10) that radiotracers may alter the charge on the cell membrane, change the polar properties of the lipoids in the membrane, and denature cellular proteins, thus invalidating experiments conducted with them. There may be limited injury when high concentrations of radioelements are used (1, 54, 105, 107), but it seems to be the general experience and consensus of opinion (20, 41, 66, 85, 105, 106) that radioelements in the limited concentration normally employed as tracers have little harmful effect on living tissues.

It is a basic assumption in tracer work that all the isotopes of a given element will be used in an equivalent manner; since their outer electron configurations are the same they should have the same chemical properties. It is not strictly true that they are chemically equivalent, for their slight differences are utilized in the concentration of stable isotopes by chemical exchange reactions (146, 151). These differences are small enough, however, so that our analytical methods are usually not precise enough to establish

any biological separation of the isotopes. Thus preferential utilization of one isotope of an element does not constitute a problem in tracer work. To this general statement an exception must be made for deuterium and tritium. Deuterium with twice and tritium with three times the mass of hydrogen differ substantially from hydrogen and have distinctly different effects on biological systems. In dilute solutions D_2O has shown no influence on growth of *Aspergillus niger*, germination of conidia of *Erysiphe graminis tritici*, root growth of wheat, and oxygen consumption by wheat seedlings (42). D_2O in high concentrations has proved inhibitory to the inversion of sucrose, catalase activity, the photosynthetic reaction (43, 121) and other biological reactions (125, 126). Tritium shows even greater differences from hydrogen. However, in the concentrations sufficient for most tracer work neither D nor T should prove toxic. The problem of isotope fractionation remains in using D_2, DT, D_2O, DTO, *etc.*, but does not apply to the use of D or T as labels for a carbon chain.

ION ABSORPTION AND EXCHANGE

Isotopes are nicely suited to investigation of ion absorption and exchange, and their extensive use in this field has been very informative. Hevesy (63, 64, 65) demonstrated that radiolead was rapidly taken up by the roots of *Vicia faba*. When the roots were then immersed in a solution containing a high concentration of normal lead, most of the radiolead went back into the external solution. Copper would displace lead from the roots very effectively; cadmium, zinc, chromium, barium and sodium were distinctly less effective. This ready displacement of radiolead suggested that it was predominantly present in the roots in an inorganic dissociable form rather than in an organic complex. The absorption of lead by the plant apparently had no direct connection with transpiration. When dilute solutions of radiolead were supplied, the bulk of it was retained in the roots, whereas from more concentrated solutions proportionately larger quantities were transferred to the tops of the plants.

There have been many demonstrations of the ability of single cells and intact roots to accumulate ions against a concentration gradient. In early experiments (91) it was demonstrated that the accumulation of radiolead by *Valonia macrophysa* occurred in the

protoplasm and that very little of the ion entered the vacuole; when the cells were killed radiolead passed into the vacuole. The accumulation of uranium, radium and actinium in the roots of peas and other plants (7), copper in immersed leaves of *Elodea canadensis* (100, 101), rubidium and phosphorus by barley roots (117), and rubidium, bromine (24, 75), sodium and potassium in *Nitella* cells (22, 23, 24) has been demonstrated. In these cases, as with the accumulation of lead by *Valonia*, the concentration occurred in the protoplasm. Concentrations higher than those in the bathing fluid may be attained in the protoplasm in less than a minute (24, 25). Initially the labeled ion is more concentrated in the hyaline protoplasm, but later the granules of the protoplasm attain a higher concentration than the hyaline portion (108). The stimulation of organisms by extremely low concentrations of heavy metal ions (oligodynamic action) has been attributed to their ability to accumulate ions (101).

The initial rapid uptake of radioactive ions has been explained as an exchange of inorganic cations between the cell and the external medium (22, 24, 81). This initial exchange may be followed by the maintenance of a rather stable concentration or some loss of radioions, this phase in turn being followed by a slow but steady metabolic uptake of ions. A subsequent loss of ions may result from injury to the cells. Compared to accumulation in the protoplasm, movement of ions into the vacuole is relatively slow. After several hours exposure the concentrations in sap and protoplasm may not be greatly different (77). Tests with radiobromine (22) revealed no rapid uptake initially by *Nitella;* perhaps the cells had no appreciable concentration of proper ions for exchange.

The accumulation of ions is influenced by the energy metabolism of the plant (74). The respiration and fermentation of the plant generates H^+ and HCO_3^- which can be exchanged for ions from the nutrient medium (21). Under anaerobic conditions, or in the presence of respiratory poisons such as cyanide, accumulation of radioisotopes of rubidium or bromide is greatly inhibited (75). The initial non-metabolic exchange has a very low Q_{10} (about 1.10), whereas later accumulation has a Q_{10} of about 2.2, indicating its dependence upon respiration (109). Initially a good share of absorbed tracer ions may be held by the roots, but soon they move into the shoots as well (26).

The absorption rate for potassium ions by barley roots was found to be independent of concentration above 0.005 M, and calculations indicated that only about $1 \times 10^{-5}\%$ of the ions striking the root surface were absorbed (116). Absorption and translocation of ions by young barley was almost as active in the dark as in the light over short periods (76). Other observations with tomato have indicated a greater uptake of P^{32} as phosphate during the day than during the night and greater at 26.5° C. than at 18° C. (156).

When carrier-free Rb^{86} and P^{32} as phosphate were supplied in about 10^{-9} molar concentrations to actively metabolizing barley roots, the ions were accumulated rapidly (117). When the metabolism of the roots was virtually halted by keeping them at 0° C., the Rb^{86} absorbed non-metabolically was readily exchanged for normal rubidium added to the bathing solution, but the P^{32} taken up was not readily exchangeable. The non-metabolic uptake was largely confined to the first few millimeters of the root tip, and in this region the concentration within the tissue was greater than the external concentration of the ions; similar results were observed with carrier-free radiostrontium and radioiodine (79). In these experiments the root hairs did not appear particularly active in accumulating the radiostrontium and iodine. Roots of barley killed by treatment with ether are still able to take up strontium and iodine, and the dead roots may actually "accumulate" strontium ions. The killed tissues at 0° C. lose their isotopic tracers to a normal solution of ions much more readily than do live roots. It is suggested (79) that an ion-binding substance is elaborated in protoplasm during its aerobic metabolism.

After radioions have been taken up by plant cells they are not lost readily when the cells are immersed in distilled water (109, 110). However, they may be displaced by normal ions in the external medium (19, 110, 115, 117). It is evident that under these conditions ions pass into the cell, while others leave the cell; thus it is possible to build up or decrease the concentration of a radioion in a plant cell without the plant experiencing any shift in the total quantity of the ion present in its radioactive plus non-radioactive forms. The exchange movement of ions is much less dependent upon metabolic processes than is the accumulation of ions (27). An ion may be displaced from a cell by a non-homolo-

gous ion; thus lithium, potassium, rubidium and cesium will displace sodium; the lithium is more active in displacing sodium than is sodium itself (110).

The mechanism of absorption and loss of ions in soil is considered by some to involve an exchange between roots and soil colloids which are in contact (80, 81). After barley plants were exposed for two to five hours to radiopotassium in their nutrient solution, the roots were rinsed and placed in various nutrients without radioindicators. The radiopotassium was removed better by monovalent than by divalent cations, and better by cations adsorbed on clay particles than by cations in solution (82). This greater activity of contact exchange by adsorbed cations did not hold true for anions; radiobromine was removed from roots better by bromine in solution than by bromine adsorbed on clays. If roots and clay suspensions were separated by a semipermeable membrane, the solutions were more effective than the clay suspensions in removing radiopotassium and radiosodium.

When C^{11}-labeled $KHCO_3$ solution was supplied to barley seedlings (118), the bicarbonate ion was absorbed by the root system. However, the potassium was absorbed at a much more rapid rate. This differential absorption supports the ion exchange absorption theory (109) as opposed to the concept of absorption of intact molecules.

An influence of one ion upon another is shown by the influence of boron upon the concentration and solubility of strontium in tomato leaves (142). These experiments (142) were conducted with strontium on the assumption that it is metabolized in the same manner as is calcium; the use of strontium is justified as a qualitative but not as a quantitative tracer for calcium. The use of P^{32} has revealed that phosphate is more rapidly absorbed by phosphorus-deficient than by normal tomato plants, although it is distributed in the same compounds in each case (18). Tomato plants furnished urea absorbed phosphorus faster than plants grown on nitrate.

The problems of ion absorption and exchange are met constantly by those interested in soils. Isotopic tracers offer an elegant means for determining the origin of ions taken from the soil by plants. Fertilization is complicated by the fact that materials applied to soil may be bound by soil constituents, and as a result

there is no assurance than an element appearing in a plant arises from the fertilizer element applied rather than from the same element already present in the soil. By the use of isotopes a fertilizer element may be labeled and traced unequivocally into the plant.

Tracer studies with soils were pioneered by Ballard and Dean (5) who described a great variation in sorption of P^{32} by different soils. A series of Florida soils ranked the same in phosphorus-fixing capacity, whether determined by chemical analysis, by measurement of P^{32} in equilibrium solution or by uptake of P^{32} by oat plants (40). The fixation of phosphorus was related to the clay content of the soils examined (111). Tests (5) on the application of P^{32} at the surface, deep in the soil at root level, and uniformly mixed throughout the soil indicated the greatest uptake of P^{32} from the deep application. Field experiments indicated that wheat plants grown about three and a half months recovered 23.6% of the P^{32} added to the soil (9, 143, 144). Plants vary greatly in their ability to utilize "fixed" phosphorus of the soil (6); Sudan grass, for example, uses it more readily than tomatoes.

P^{32} as phosphate and radiopotassium washed into the soil with the equivalent of two and a half inches of rainfall penetrated into a variety of soils to a depth of only an inch or two (62). As grape vines had not shown any measurable response to phosphorus fertilization, P^{32}-labeled phosphate was used to test their assimilation of the element. P^{32} plus normal carrier phosphate added at a 15-inch depth was taken up more quickly and in about twice as great an amount as when added to the surface; both treatments were followed by heavy irrigation. After 11 days, 99% of the P^{32} applied at the surface was in the top six inches of soil (149).

The fixation of cations by soil may be altered by grinding the soil. RbCl was added to soils, and the soils were air dried and then leached with ammonium acetate to remove replaceable Rb. By thorough regrinding in a ball mill Rb was rendered exchangeable to the extent of 27% for one and 50% for another soil, as measured by a repetition of the leaching process (99).

The availability of nitrogen from decaying organic matter has been tested by growing soybeans on a N^{15}-enriched nitrogen source, grinding the plants and incorporating them into soil-sand mixtures (113). Soybeans were grown on this soil for 11 weeks and

tested for their N^{15} content; this was a measure of the proportion of their nitrogen derived from that originally present in the soil and that arising from decomposition of the added plant residues. Soybean plants which were inoculated (total nitrogen, 2,148 mg.) picked up 283 mg., and uninoculated plants (total nitrogen, 912 mg.) picked up 282 mg. of nitrogen from the plant residues in the soil.

TRANSPORT OF NUTRIENTS

Hevesy's early experiments (63) indicated that readily measurable quantities of radiolead were transported from the roots to the stems and leaves of *Vicia faba* plants, even when concentrations of lead nitrate as low as 1×10^{-6} N were supplied in the nutrient solution. At this concentration about 60% of the lead was taken up from the solution within 24 hours; at 0.1 N only 0.3% was taken up. At low concentrations a great percentage of the lead was bound in the root, whereas at higher concentration a proportionately greater amount was transported to the tops.

Following the earliest reports on the transport of P^{32} in plants (65, 70) there have been several studies of the transport of this excellent tracer. When part of a root system is immersed in P^{32}-labeled phosphate and part in normal phosphate, the transfer of P^{32} to the roots bathed in normal phosphate is rather slow (68). P^{32} was detected in the leaves of red Mexican bean seedlings two and a half hours after its addition as phosphate to the nutrient solution; the rate of transport exceeded ten centimeters an hour (13). The P^{32} accumulated in the roots to rather high concentrations before moving to the tops (14). The migration of P^{32} from the point at which it was injected into bean leaves seemed to follow a diurnal cycle with greatest downward movement near 10 A.M., the least at 10 P.M. and the maximum upward movement near noon (15). In a variety of plants both the xylem and phloem appeared to function in the upward transport of minerals (58). In the cotton plant radiophosphorus moved from the leaf via the phloem in both an upward and downward direction. The rate of downward movement was greater than 21 cm. per hour (16).

The presence of P^{32} in the tips of six-foot tomato plants was observed 40 minutes after the radioisotope was added to the nutrient

solution (2). Radioautographs indicated that the P^{32} was absorbed in considerable quantity by the pulp and seeds of green tomatoes, a tendency that decreased with ageing of the fruit. On a limited supply of phosphorus the plant withdrew phosphorus from the leaves and transferred it to the developing fruit.

Complete sections of xylem were removed from geranium and *Bryophyllum calycinum* plants, and then the roots of the plants were immersed in a labeled phosphate solution. The concentration of P^{32} in the tops after 17 to 40 hours was about the same with intact as with treated plants, indicating that phosphate is transported in the pholem (57). These apparently clear-cut data have been challenged because of the relatively long time which elapsed before samples were taken.

When an attempt was made (39) to confine the transport of P^{32} to the phloem of Hubbard squash plants, the movement of P^{32} seemed to be correlated with the movement of food in the plants. In other experiments phosphate containing P^{32} was applied to the leaves after the petioles had been scalded to render the phloem non-conducting; no P^{32} was transported from the leaves, even though the xylem was functional. However, if the leaves were flooded with liquid, P^{32} and considerable liquid were transported through the scalded petioles. Three hours after vacuum infiltration of P^{32} into the leaves of intact plants the radioelement could be found 90 cm. from the point of treatment. Transport was through the xylem, as it was not prevented by scalding the petioles. Additional studies on the transport of radiophosphorus (19, 147) and radiosodium (19, 112, 147) have shown that both elements pass rapidly in a lateral direction from wood to bark (147); the indications are that the xylem is the path of rapid upward movement of salt.

Radiosulfur supplied in the nutrient solution to sulfur-deficient wheat and barley initially was accumulated avidly by the leaves; later this sulfur was more uniformly distributed throughout the plant (148). The bulk of the sulfur moved to the grain during ripening. Sulfur dioxide labeled with radiosulfur was used to fumigate plants. Again the radiosulfur accumulated in the leaves, and, as when sulfate was supplied in the nutrient solution, the inorganic forms were rapidly incorporated into organic compounds. Corn plants which had been given adequate amount of sulfur did

not take up the labeled sulfate nearly as rapidly as the deficient wheat and barley. Radioautographs (61) indicated that radiosulfur was rather uniformly distributed in the wheat leaf, but in the kernel there was a marked concentration of sulfur in the embryo and also in the periphery of the endosperm. The distributions of protein and radiosulfur were very similar.

The stable isotope C^{13} has been employed to trace the transport of the products of photosynthesis (123). When single leaves were enclosed, furnished with $C^{13}O_2$ and illuminated, the photosynthate carrying the C^{13} label was transported both up and down the stem and tended to accumulate at the points of most active growth. After killing a section of stem with hot wax, P^{32} as phosphate passed up the stem, whereas C^{13}-labeled photosynthate did not pass up or down through such a block. This suggested the phloem as the exclusive path of transport for the photosynthate.

PHOTOSYNTHESIS

The importance and complexity of the photosynthetic reactions present a constant challenge. Isotopic tracers have been extremely useful in extending our knowledge of this process.

Results of a series of experiments with the short-lived radiocarbon, C^{11}, led Ruben and co-workers at Berkeley to conclude that the first measurable product of photosynthesis is a complex compound rather than any of the simple ones suggested earlier. Products of the dark fixation of $C^{11}O_2$ by *Chlorella pyrenoidosa* carried most of the C^{11} in material precipitable by barium in 80% ethyl alcohol (135); virtually all of this C^{11} was present in carboxyl groups. Dark fixation of CO_2 was also observed with barley leaves that had been illuminated shortly before exposure to $C^{11}O_2$ in the dark (130).

When the complete photosynthetic reaction occurred in the light, $C^{11}O_2$ was fixed in –CHOH– groups as well as in carboxyl groups (134). An examination by the carrier technique for products of photosynthesis after exposure of *Chlorella* to $C^{11}O_2$ for very short periods, yielded no evidence for the following substances as early products of photosynthesis: formaldehyde, acetaldehyde, propionaldehyde, glycolic aldehyde, glyceric aldehyde, methanol, ethanol, glycol, glycerol, erythritol, glucose, sucrose, starch, hexose monophosphate, glycine, alanine, arginine, histidine, albumin, acetone,

and the acids formic, acetic, propionic, butyric, oxalic, succinic, malic, citric, maleic, fumaric, glycolic, pyruvic, glyceric, tartaric, lactic, ascorbic, glucuronic, glutamic, aspartic and glutaric.

The photosynthetic product from *Chlorella pyrenoidosa* carrying the bulk of the C^{11} had a molecular weight in the range of 1,000 (136), as established by ultracentrifugation. It was proposed (132) that photosynthesis occurs by an initial dark carboxylation of a high molecular weight compound to give RCOOH and that this is followed by a reduction of the carboxyl group in a light reaction to yield RCHO and then RCH_2OH with simultaneous liberation of O_2 from H_2O. Subsequent carboxylations and reductions could build up carbohydrate chains which could be split from the compound of high molecular weight.

When C^{14}-labeled acetate is supplied to tobacco leaves, a part of that which is not respired is soon converted to a wide variety of compounds (89). Tests with sunflower leaves have indicated that the photosynthetic products are rather rapidly used in respiration (141).

Experiments with Mg^{27} have yielded no support for the postulate that a reversible oxidation reduction occurs between chlorophylls a and b in photosynthesis (129). Tritium, H^3, was employed to test whether chlorophyll acts as a hydrogen donor in photosynthesis by illuminating *Chlorella pyrenoidosa* in HTO plus H_2O, isolating chlorophyll from the cells and testing it for radiohydrogen. Although the experiments are open to the possible objection that isotope fractionation does not favor T uptake, still they are highly suggestive that chlorophyll does not function as a hydrogen donor (114).

The origin of O_2 in the photosynthetic reaction was long a subject of speculation. Its origin remained undetermined until use of the stable isotope O^{18} revealed that O_2 arises exclusively from water (137). *Chlorella* cells photosynthesizing the O^{18}-enriched water evolved O_2 with the same O^{18} content as that of the water. The observation has been verified (44) and also shown to hold true for the Hill reaction (78).

Attempts to induce photosynthesis by ruptured *Nitella* cells were unsuccessful both with the chloroplastic and non-chloroplastic fractions (50). The products from photosynthesis by intact *Nitella* in the presence of $C^{11}O_2$ were water-soluble.

The availability of C^{14} recently has given new impetus to investigations of the early products of photosynthesis. Gaffron and co-workers (51) have substantiated the observation that a rather high molecular weight compound of unique properties is an early product of photosynthesis and that the commonly postulated intermediates do not appear to be involved. They have isolated a fraction, soluble in hot alcohol and insoluble in benzene, from *Scenedesmus* cells exposed to $C^{14}O_2$ for a minute or less; this fraction composing less than 1% of the dry weight of the algal cells carries the bulk of their radioactivity. Characterization of the compound is incomplete, but it has carboxyl and hydroxyl groups, may have lactone rings and is of rather high molecular weight.

In contrast, Calvin and co-workers (12, 37, 38) have studied the dark reaction in *Chlorella pyrenoidosa* and *Scenedesmus* with the assumption that the dark reaction is in all likelihood the initial step in photosynthesis and a logical point for isolating first products of the reaction. Isolations from *Chlorella* cells, previously treated to favor accumulation of reducing products and then exposed for short periods to $C^{14}O_2$, have shown high concentrations of the isotope in malic acid and in alanine. The high C^{14} specific activity of the phosphoglyceric acid and triosephosphate fractions suggests the condensation of triosephosphates as a likely pathway for sugar synthesis (38). It is tentatively proposed that carboxylations similar to those involved in the formation of pyruvic, oxalacetic and oxalosuccinic acids are concerned in photosynthesis. This viewpoint has been challenged and the data interpreted as indicative of "respiratory" CO_2 fixation quite apart from any function in photosynthesis. The two groups of investigators are operating under distinctly different experimental conditions, and it is too early to judge the validity of their respective conclusions regarding the early products of photosynthesis. Certainly the current efforts should greatly expand our information on the photosynthetic reaction.

The distribution of C^{14} in photosynthesizing barley seedlings has been determined (4). Glucose isolated from barley plants supplied with $C^{14}O_2$ showed the highest C^{14} concentration in the 3 and 4 positions and the least in the 1 and 6 positions. The distribution of C^{14} in glucose from intact plants was distinctly different from

that in plants without roots. The data are compatible with the idea that hexoses are formed from triose phosphates and that the terminal carbons of the trioses, which become carbons 3 and 4 of the hexose, have particularly high C^{14} activity (3).

HETEROTROPHIC FIXATION OF CARBON DIOXIDE

Heterotrophic fixation of carbon dioxide has been shown to occur in plant and animal tissues and bacteria (133, 160). Barley roots assimilated $C^{11}O_2$ (133). A specific system involved in such CO_2 assimilation has been obtained in a cell-free state from parsley roots (153). The formation of oxalosuccinic acid from alpha-ketoglutaric acid had been demonstrated earlier with animal tissue as a reversible step in the tricarboxylic acid cycle. It is not unlikely, in fact evidence suggests, that a cycle such as that found in animal tissue also occurs in plants. However, except for the experimental work cited (153), the data presented in support of a tricarboxylic acid cycle in plants are non-specific and scarcely convincing. A thorough investigation of possible intermediates, specific steps and the overall reaction with the aid of isotopically labeled compounds is needed for our acceptance or rejection of the cycle in plants.

BIOLOGICAL NITROGEN FIXATION

The radioactive forms of nitrogen have very short half-lives; the half-life of N^{13} is 9.9 minutes. This limits their usefulness, and the stable isotope, N^{15}, is much more satisfactory as a tracer.

It was observed (131) that the tops of barley plants exposed for 20 minutes to N_2 containing N^{13} picked up radioactivity with the N^{13} half-life. This was surprising, since non-leguminous plants are not generally credited with the ability to fix molecular nitrogen. Experiments with N^{15} indicated that no exchange reaction of appreciable magnitude occurred in the biological fixation of nitrogen (33). The N^{13} experiment was repeated (29), using N^{15} as the tracer and growing intact barley plants aseptically for periods of 42 and 56 days; inoculated and uninoculated clover plants were subjected to the same treatment. No fixation of nitrogen greater than the experimental error of the method was observed for barley or for uninoculated clover, whereas the inoculated clover plants fixed nitrogen actively. Calculations indicated that the discrepancy in results could not be attributed to the greater sensitivity of the radioactive tracer.

Numerous agents have been alleged to fix molecular nitrogen, and many of these claims have now been checked with N^{15}; N^{15} offers a much more sensitive and specific means of testing such claims than does the Kjeldahl nitrogen determination. The well established nitrogen-fixing agents, leguminous plants plus root nodule bacteria (*29*), the aerobic *Azotobacter vinelandii*, the anaerobic *Clostridium pasteurianum* and the blue-green alga *Nostoc muscorum*, all fixed large quantities of N^{15} (*32*). The questionable agents, aseptic germinating peas, free-living root nodule bacteria, cell-free preparations of *Azotobacter* (*32*) and ground pea plants (*31*), gave uniformly negative results. Excised nodulated roots from leguminous plants fixed nitrogen rather consistently, but excised nodules only occasionally (*32, 98*). In contradistinction to carbon dioxide fixation, which isotopic tracers have revealed to be widespread in nature, biological nitrogen fixation is apparently limited to relatively few living forms.

The root nodules have been accepted as the actual locus of nitrogen fixation in leguminous plants. Experimental proof of this point has been rather difficult to obtain, but the accepted idea has been verified by the use of N^{15} (*31*). Although much larger amounts of N^{15} were fixed when it was supplied to the roots, it is interesting that when N^{15}-enriched N_2 was supplied to the tops of the plants it could be detected in small amounts in the nodules. This indicates that in all probability N_2 in solution passed through an exposed section of stem through the root and into the nodule where it was fixed.

N^{15} has been useful in establishing certain of the physical constants characteristic of the nitrogen-fixing enzymes of the blue-green alga, *Nostoc muscorum* (*36*).

Experimental data accumulated on biological nitrogen fixation with N^{15} as a tracer have been summarized and evaluated recently (*158*). It has been possible to study the relative use of fixed nitrogen compounds and molecular nitrogen by supplying *A. vinelandii* with fixed nitrogen compounds containing normal nitrogen in the nutrient medium and N^{15}-enriched N_2 in the atmosphere above the medium. The organism can obtain its nitrogen exclusively from the fixed nitrogen or from the N_2 or can obtain part from each—final analysis of the cells indicates how much of their nitrogen came from each source. Such experiments have indicated that only ammonia and urea (urea is practically equivalent to

ammonia because of the strong urease in the cells) completely suppress nitrogen fixation in cultures which have not previously been grown on fixed nitrogen. Nitrate and nitrite suppress fixation considerably; amino acids alone or in mixture only slightly (159).

If $(N^{15}H_4)_2SO_4$ is added to an aerated culture of *A. vinelandii* which has been fixing N_2 as its sole source of nitrogen, the fixation of N_2 is immediately (within one minute) and completely suppressed (35). When $N^{15}O_3^-$ is added there is no detectable uptake of N^{15} for 30 minutes, after which nitrate is assimilated at an accelerating rate. When $N^{15}H_4NO_3$ is added to cultures of *A. vinelandii* which have been grown previously on N_2, the ammonium ion is used up before the nitrate is assimilated. Even cultures which have been grown on KNO_3 up to the time of the addition of $N^{15}H_4NO_3$ use the ammonium before the nitrate ion (35). Apparently *A. vinelandii* cells which are dependent upon N_2 (or NO_3^-) as their sole source of nitrogen have all the enzymes necessary for the utilization of the ammonium ion to the exclusion of other nitrogenous compounds, whereas the cells must adapt themselves to utilize nitrate.

A. vinelandii cells were grown in aerated culture for 18 hours and then aerated for 90 minutes with O_2 and N^{15}-enriched N_2. Certain amino acids were isolated from the cells, and analysis revealed that the highest N^{15} concentration was in glutamic acid, the next highest in the aspartic acid fraction (30). Likewise when N^{15}-labeled ammonium ion was added to an aerated culture the glutamic and aspartic acids carried the highest concentration of N^{15}, and the distribution of N^{15} in the other compounds was very similar to that found when N^{15}-enriched N_2 was supplied (34).

All the data from N^{15} experiments with leguminous plants and the free-living nitrogen-fixing bacteria appear compatible with the ammonia hypothesis of biological nitrogen fixation. Further application of this tracer should give us greater insight into the intermediates in the fixation reaction and additional suggestions on the mechanism of the reaction.

METABOLISM OF NITROGEN IN PLANTS

Other than in studies of biological nitrogen fixation, N^{15} has received rather limited use in investigations of the nitrogen metabolism of plants. The administration of N^{15} as ammonia to buckwheat (154), to tobacco plants (155) and to sunflower (69) in-

dicated that there was a rapid replacement of leaf protein nitrogen by simple nitrogenous compounds of the cell sap and that a continuous exchange occurred between free amino acids and those combined in proteins. This established in plants, as had been demonstrated earlier with animals, that a dynamic equilibrium exists among nitrogenous compounds. The demonstration with plants is necessarily less elegant than with animals because plants cannot be maintained in a condition of nitrogen balance as can animals.

METABOLISM OF PHOSPHORUS IN PLANTS

In addition to studies of ion absorption and transport in plants, P^{32} has been used to establish the mobility of the element during growth of the plant. After ten days in a culture solution, corn plants were transferred to a solution containing P^{32} as phosphate. The plants grew an additional 21 days, were harvested, ashed and analyzed for P^{32}. The lower leaves, which were almost entirely formed before P^{32} was added, had virtually the same concentration of P^{32} as had the new leaves formed entirely after the addition of P^{32} (70). Very similar results were observed with sunflower (71). It is evident that phosphorus is a highly mobile element in the plant, and the newly acquired phosphorus readily exchanges with that laid down earlier in the plant.

PHYTOPATHOLOGY

By growing tobacco in a nutrient solution containing P^{32} it has been possible to isolate tobacco mosaic virus with good recovery of the P^{32} (17, 94, 145). When this virus labeled with P^{32} was inoculated into leaves and allowed to remain for 12 days, the bulk of the P^{32} was recovered in non-virus components. The difficulties in using labeled virus were enumerated (145).

There was little difference in the total uptake of P^{32} by healthy and heavily rusted wheat leaves, but in the rusted leaves the P^{32} accumulated in the infected areas. This concentration of P^{32} likely occurred in the host cells, since the uredospores carried only low levels of P^{32} (55).

PLANT-GROWTH REGULATORS

The transport of herbicides has been followed very nicely by the use of I^{131} as a label. This isotope is an excellent tracer, for it can be obtained in high specific activity, has a reasonable half life, 8.0

days, and emits energetic radiations. I^{131} was incorporated into 2-iodo-3-nitrobenzoic acid and followed through bean and barley plants. The compound applied to the leaves of bean seedlings was translocated to the stems and accumulated mainly in the terminal buds and in the hypocotyls. When absorbed by barley leaves it accumulated mainly in the second leaf (161); the growth regulator was translocated somewhat less readily than in bean. Further studies (104) again indicated that oat and corn plants absorbed less of the growth regulator than did bean plants. However, this observation could not account for the differential effects of the compound on mono- and dicotyledonous plants, for at equal concentrations of 2-iodo-3-nitrobenzoic acid in the plant tissues the bean plants showed marked responses, whereas the corn and oat plants were unaffected.

In experiments such as those just described it is dangerous to assume that when 2-iodo^{131}-3-nitrobenzoic acid is applied to a leaf and I^{131} is found in another part of the plant, 2-iodo^{131}-3-nitrobenzoic acid is present in this other part of the plant; it is always possible that the compound may be broken down and the I^{131} transported in another form. Fortunately this possibility has not been overlooked, and data have been presented (103) to establish that in these tests the intact herbicide molecule migrated.

STIMULATION OF THE GROWTH OF PLANTS BY RADIOACTIVE ELEMENTS

Hevesy and Paneth (73) refer to early work which indicated that, although large doses of radium are harmful, small amounts distinctly improve the growth of plants. Gager (52, 53) observed stimulation of plant growth under certain conditions by Ra but was not convinced of its practical importance. Over 100% increase in the dry weight of pea plants has been reported when 1×10^{-11} g. of radium was added to six liters of nutrient solution; even 1×10^{-8} g. per six liters was not lethal (45). The conclusion is drawn that uranium, radium, actinium and thorium are necessary nutrients for plants and that potassium cannot replace other radioactive elements; the interchangeability of radium, thorium and uranium was not tested. Although some other stimulations (11) have been reported, the lack of control and re-

plication in the experiments suggests need for their verification. Extensive tests of the claims are currently planned in this country; some of the preliminary tests on vegetable crops under field conditions here have shown distinct stimulation by radioisotopes. Additional reports have been published concerning the stimulation of plant growth by radioactivity (46), the influence of radioactivity on nodulation of leguminous plants (47), the assimilation of naturally occurring radioelements (7), the amount of naturally occurring radioelements in soils (8) and the influence of immersion of plants in radioactive spring water upon their subsequent growth and development (93).

POTENTIAL USES OF ISOTOPES AS TRACERS

The foregoing treatment necessarily has been sketchy with regard to any particular application of isotopic tracers, but the variety of problems in which they have yielded valuable information is suggestive of their potential future usefulness. Many problems not amenable to the classical chemical or physiological approach may be attacked successfully with the aid of isotopes.

Isotopes are elegantly suited for investigations of ion absorption, exchange and transport in the plant, and unquestionably they will be used extensively in such work. It is hoped that accompanying such studies there will be more emphasis on the elucidation of the specific function of the various mineral elements in plant metabolism.

Many problems in soil chemistry are ideally suited for the use of isotopes. At last a means is available to distinguish fertilizer elements added to the soil from those already present and to determine their respective rôles in the nutrition of the plant. Studies on the influence of various treatments upon the mobilization of bound elements in the soil and upon the function of microorganisms in this regard could be conducted readily with isotopic tracers.

There is no question but that isotopes will be of immense aid in untangling some of our most complex plant processes, such as photosynthesis and biological nitrogen fixation. Already encouraging progress has been made in determining the early products of the photosynthetic reaction, and information obtained with

radiocarbon has forced us to discard postulated intermediates which have beclouded the photosynthetic picture for three quarters of a century.

With a disappearance of the early tendency to use isotopes for the sake of employing a fashionable technique rather than for the inherent value they might have for the problem at hand, it is anticipated that more solidly conceived and carefully executed work will be forthcoming. Studies involving the isolation and characterization of intermediates will be helpful in establishing the mechanism of respiration in plants, the occurrence or non-occurrence of an organic acid cycle in plants, and the mechanism of synthesis of carbohydrates, proteins and lipids.

SUMMARY

The recent release of radioisotopes as produced in the uranium pile, the availability of reliable equipment for measuring radioactivity, and the commercial concentration of stable isotopes have given great impetus to the use of isotopes as tracers. The radioactive isotopes excel the stable isotopes in ease of measurement, variety available, dilution in which they can be detected, and for detection in intact organisms, whereas the stable isotopes are favored for their stability, applicability for tracing H, N and O, and their lack of radiation hazard. However, the stable and radioactive isotopes should be considered as complementary tools.

Radioisotopes have revealed the great speed of ion penetration into cells, the ready exchangeability of ions inside and outside the cell, and the dependence upon respiration for ion accumulation subsequent to the initial ion exchange.

Isotopes offer a means for differentiating added fertilizer elements from those already present in soils in the investigation of the nutrition of plants. In addition they offer an excellent means for following the transport of elements and compounds in plants.

Application of C^{11} and C^{14} has forced the discarding of old hypotheses concerning the early products of photosynthesis. Two distinct postulates have replaced the earlier ideas, one favoring the early formation of a hydroxylated, carboxyl compound of high molecular weight, and the other implicating the organic acids, particularly malic, as early products of photosynthesis. With the aid of O^{18} it has been shown that H_2O is the exclusive source of O_2 in the photosynthetic reaction.

Whereas the use of isotopes of carbon has revealed the widespread occurrence of heterotrophic fixation of carbon dioxide, the use of N^{15} has confirmed the limited distribution among organisms of the ability to fix N_2. Data obtained with N^{15} have all been compatible with the ammonia hypothesis of biological nitrogen fixation. As in animals, the nitrogenous constituents of plants are not inert but are in dynamic equilibrium.

Phosphorus is a highly mobile element in the plant, as evidenced by the uniform distribution of P^{32} in old and young leaves of plants supplied P^{32} when nearing maturity. P^{32} has been used in investigating the changes which occur in tobacco mosaic virus injected into leaves.

The movement of herbicides in plants has been traced with I^{131}, and it has been demonstrated that the differential response of mono- and dicotyledonous plants cannot be explained merely on the basis that the monocotyledonous plants absorb more of the herbicide.

Literature has been cited advancing the claim that radioactivity stimulates the growth of plants, but the data are in need of extension and verification.

NOTE ADDED IN PROOF

Many notable publications describing applications of isotopes to studies of reactions in plants have appeared since this review was transmitted July 27, 1948. Particular progress has been made in the investigation of photosynthesis with C^{14} as a tracer. Extensive collaborative experiments with P^{32} have revealed numerous new facts on the binding and recovery of this element from soils. Whereas early tests suggested that addition of radioisotopes enhances the yield of certain truck crops, subsequent trials have been predominantly negative.

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